JOINT INVENTORS

"EXPRESS MAIL" mailing label No. EL564458923US.
Date of Deposit: June 26, 2001
I hereby certify that this paper (or fee) is being deposited with the United States Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 CFR §1.10 on the date indicated above and is addressed to: Commissioner for Patents, Washington, D.C. 20231

Richard Zimmermann

APPLICATION FOR UNITED STATES LETTERS PATENT

S P E C I F I C A T I O N

TO ALL WHOM IT MAY CONCERN:

Be it known that we, W. Michael Gallatin a citizen of the United States of America, residing at 8412 SE 33rd Place, Mercer Island, 98040, in the State of Washington and Monica Van der Vieren a citizen of the United States of America, residing at 2446 NW 64th Street, Seattle, 98107, in the State of Washington have invented a new and useful NOVEL HUMAN β_2 INTEGRIN ALPHA SUBUNIT, of which the following is a specification.

Novel Human β_2 Integrin Alpha Subunit

This application is a continuation-in-part of U.S. Patent Application Serial No: 08/943,363 filed October 3, 1997, which is pending, which a continuation-in-part of U.S. Patent Application Serial No. 08/605,672, filed February 22, 1996, which issued as U.S. Patent 5,817,515 on October 6, 1998, which is a continuation-in-part of U.S. Application Serial No. 08/362,652, filed December 21, 1994, which issued as U.S. Patent 5,766,850 on June 16, 1998, which is a continuation-in-part of U.S. Application Serial No. 08/286,889, filed August 5, 1994, which issued as U.S. Patent 5,470,953 on November 28, 1995, which in turn is a continuation-in-part of U.S. Application Serial No. 08/173,497, filed December 23, 1993, which issued as U.S. Patent 5,437,958 on August 1, 1995.

Background of the Invention

The integrins are a class of membrane-associated molecules which actively participate in cellular adhesion. Integrins are transmembrane heterodimers comprising an α subunit in noncovalent association with a β subunit. To date, at least fourteen α subunits and eight β subunits have been identified [reviewed in Springer, *Nature 346*:425-434 (1990)]. The β subunits are generally capable of association with more than one α subunit and the heterodimers sharing a common β subunit have been classified as subfamilies within the integrin population.

One class of human integrins, restricted to expression in white blood cells, is characterized by a common β_2 subunit. As a result of this cell-specific expression, these integrins are commonly referred to as the leukocyte integrins, Leu-CAMs or leukointegrins. Because of the common β_2 subunit, an alternative designation of this class is the β_2 integrins. The β_2 subunit (CD18) has previously been isolated in association with one of three distinct α subunits, CD11a, CD11b or CD11c. The isolation of a cDNA encoding human CD18 is described in Kishimoto, *et al.*, *Cell 48*:681-690 (1987). In official WHO nomenclature, the heterodimeric proteins are referred to as CD11a/CD18, CD11b/CD18, and CD11c/CD18; in common nomenclature they are referred to as LFA-1, Mac-1 or Mo1 and p150,95 or LeuM5, respectively [Cobbold, *et al.*, in *Leukocyte Typing*

III, McMichael (ed), Oxford Press, p.788 (1987)]. The human β_2 integrin α subunits CD11a, CD11b and CD11c have been demonstrated to migrate under reducing condition in electrophoresis with apparent molecular weights of approximately 180 kD, 155 kD and 150 kD, respectively, and DNAs encoding these subunits have been cloned [CD11a, Larson, *et al.*, *J.Cell Biol. 108*:703-712 (1989); CD11b, Corbi, *et al.*, *J.Biol.Chem. 263*:12403-12411 (1988) and CD11c, Corbi, *et al. EMBO J. 6*:4023-4028 (1987)]. Putative homologs of the human β_2 integrin α and β chains, defined by approximate similarity in molecular weight, have been variously identified in other species including monkeys and other primates [Letvin, *et al.*, *Blood 61*:408-410 (1983)], mice [Sanchez-Madrid, *et al.*, *J.Exp.Med. 154*:1517 (1981)], and dogs [Moore, *et al.*, *Tissue Antigens 36*:211-220 (1990)].

The absolute molecular weights of presumed homologs from other species have been shown to vary significantly [see, *e.g.*, Danilenko *et al.*, *Tissue Antigens 40*:13-21 (1992)], and in the absence of sequence information, a definitive correlation between human integrin subunits and those identified in other species has not been possible. Moreover, variation in the number of members in a protein family has been observed between different species. Consider, for example, that more IgA isotypes have been isolated in rabbits than in humans [Burnett, *et al.*, *EMBO J. 8*:4041-4047 (1989) and Schneiderman, *et al.*, *Proc.Natl.Acad.Sci.(USA) 86*:7561-7565 (1989)]. Similarly, in humans, at least six variants of the metallothionine protein have been previously identified [Karin and Richards, *Nature 299*:797-802 (1982) and Varshney, *et al.*, *Mol.Cell.Biol. 6*:26-37, (1986)], whereas in the mouse, only two such variants are in evidence [Searle, *et al.*, *Mol.Cell.Biol. 4*:1221-1230 (1984)]. Therefore, existence of multiple members of a protein family in one species does not necessarily imply that corresponding family members exist in another species.

In the specific context of β_2 integrins, in dogs it has been observed that the presumed canine β_2 counterpart to the human CD18 is capable of dimer formation with as many as four potentially distinct α subunits [Danilenko, *et al.*, *supra*]. Antibodies generated by immunizing mice with canine splenocytes resulted in monoclonal antibodies which immunoprecipitated proteins tentatively designated as canine homologs to human CD18, CD11a, CD11b and CD11c based mainly on similar, but not identical, molecular

weights. Another anti-canine splenocyte antibody, Ca11.8H2, recognized and immunoprecipitated a fourth α-like canine subunit also capable of association with the β_2 subunit, but having a unique molecular weight and restricted in expression to a subset of differentiated tissue macrophages.

Antibodies generated by immunization of hamsters with murine dendritic cells resulted in two anti-integrin antibodies [Metlay, *et al., J.Exp.Med. 171*:1753-1771 (1990)]. One antibody, 2E6, immunoprecipitated a predominant heterodimer with subunits having approximate molecular weights of 180 kD and 90 kD in addition to minor bands in the molecular weight range of 150-160 kD. The second antibody, N418, precipitated another apparent heterodimer with subunits having approximate molecular weights of 150 kD and 90 Kd. Based on cellular adhesion blocking studies, it was hypothesized that antibody 2E6 recognized a murine counterpart to human CD18. While the molecular weight of the N418 antigen suggested recognition of a murine homolog to human CD11c/CD18, further analysis indicated that the murine antigen exhibited a tissue distribution pattern which was inconsistent with that observed for human CD11c/CD18.

The antigens recognized by the canine Ca11.8H2 antibody and the murine N418 antibody could represent a variant species (*e.g.*, a glycosylation or splice variant) of a previously identified canine or murine α subunit. Alternatively, these antigens may represent unique canine and murine integrin α subunits. In the absence of specific information regarding primary structure, these alternatives cannot be distinguished.

In humans, CD11a/CD18 is expressed on all leukocytes. CD11b/CD18 and CD11c/CD18 are essentially restricted to expression on monocytes, granulocytes, macrophages and natural killer (NK) cells, but CD11c/CD18 is also detected on some B-cell types. In general, CD11a/CD18 predominates on lymphocytes, CD11b/CD18 on granulocytes and CD11c/CD18 on macrophages [see review, Arnaout, *Blood 75*:1037-1050 (1990)]. Expression of the α chains, however, is variable with regard to the state of activation and differentiation of the individual cell types [See review, Larson and Springer, *Immunol.Rev.* 114:181-217 (1990).]

The involvement of the β_2 integrins in human immune and inflammatory responses has been demonstrated using monoclonal antibodies which are capable of blocking β_2 integrin-associated cell adhesion. For example, CD11a/CD18, CD11b/CD18

and CD11c/CD18 actively participate in natural killer (NK) cell binding to lymphoma and adenocarcinoma cells [Patarroyo, *et al., Immunol.Rev.* 114:67-108 (1990)], granulocyte accumulation [Nourshargh, *et al., J.Immunol. 142*:3193-3198 (1989)], granulocyte-independent plasma leakage [Arfors, *et al., Blood 69*:338-340 (1987)], chemotactic response of stimulated leukocytes [Arfors, *et al., supra*] and leukocyte adhesion to vascular endothelium [Price, *et al., J.Immunol. 139*:4174-4177 (1987) and Smith, *et al., J.Clin.Invest. 83*:2008-2017 (1989)]. The fundamental role of β_2 integrins in immune and inflammatory responses is made apparent in the clinical syndrome referred to as leukocyte adhesion deficiency (LAD), wherein clinical manifestations include recurrent and often life threatening bacterial infections. LAD results from heterogeneous mutations in the β_2 subunit [Kishimoto, *et al., Cell 50*:193-202 (1987)] and the severity of the disease state is proportional to the degree of the deficiency in β_2 subunit expression. Formation of the complete integrin heterodimer is impaired by the β_2 mutation [Kishimoto, *et al., supra*].

Interestingly, at least one antibody specific for CD18 has been shown to inhibit human immunodeficiency virus type-1 (HIV-1) syncytia formation *in vitro*, albeit the exact mechanism of this inhibition is unclear [Hildreth and Orentas, *Science 244*:1075-1078 (1989)]. This observation is consistent with the discovery that a principal counterreceptor of CD11a/CD18, ICAM-1, is also a surface receptor for the major group of rhinovirus serotypes [Greve, *et al., Cell 56*:839 (1989)].

The significance of β_2 integrin binding activity in human immune and inflammatory responses underscores the necessity to develop a more complete understanding of this class of surface proteins. Identification of yet unknown members of this subfamily, as well as their counterreceptors, and the generation of monoclonal antibodies or other soluble factors which can alter biological activity of the β_2 integrins will provide practical means for therapeutic intervention in β_2 integrin-related immune and inflammatory responses.

Brief Description of the Invention

In one aspect, the present invention provides novel purified and isolated polynucleotides (*e.g.*, DNA and RNA transcripts, both sense and anti-sense strands) encoding a novel human β_2 integrin α subunit, α_d, and variants thereof (*i.e.*, deletion,

addition or substitution analogs) which possess binding and/or immunological properties inherent to α_d. Preferred DNA molecules of the invention include cDNA, genomic DNA and wholly or partially chemically synthesized DNA molecules. A presently preferred polynucleotide is the DNA as set forth in SEQ ID NO: 1, encoding the polypeptide of SEQ ID NO: 2. Also provided are recombinant plasmid and viral DNA constructions (expression constructs) which include α_d encoding sequences, wherein the α_d encoding sequence is operatively linked to a homologous or heterologous transcriptional regulatory element or elements.

Also provided by the present invention are isolated and purified mouse and rat polynucleotides which exhibit homology to polynucleotides encoding human α_d. A preferred mouse polynucleotide is set forth in SEQ ID NO: 52; a preferred rat polynucleotide is set forth in SEQ ID NO: 54.

As another aspect of the invention, prokaryotic or eukaryotic host cells transformed or transfected with DNA sequences of the invention are provided which express α_d polypeptide or variants thereof. Host cells of the invention are particularly useful for large scale production of α_d polypeptide, which can be isolated from either the host cell itself or from the medium in which the host cell is grown. Host cells which express α_d polypeptide on their extracellular membrane surface are also useful as immunogens in the production of α_d-specific antibodies. Preferably, host cells transfected with α_d will be co-transfected to express a β_2 integrin subunit in order to allow surface expression of the heterodimer.

Also provided by the present invention are purified and isolated α_d polypeptides, fragments and variants thereof. Preferred α_d polypeptides are as set forth in SEQ ID NO: 2. Novel α_d products of the invention may be obtained as isolates from natural sources, but, along with α_d variant products, are preferably produced by recombinant procedures involving host cells of the invention. Completely glycosylated, partially glycosylated and wholly de-glycosylated forms of the α_d polypeptide may be generated by varying the host cell selected for recombinant production and/or post-isolation processing. Variant α_d polypeptides of the invention may comprise water soluble and insoluble α_d polypeptides including analogs wherein one or more of the amino acids are deleted or replaced: (1) without loss, and preferably with enhancement, of one or more

biological activities or immunological characteristics specific for α_d; or (2) with specific disablement of a particular ligand/receptor binding or signalling function. Fusion polypeptides are also provided, wherein α_d amino acid sequences are expressed contiguously with amino acid sequences from other polypeptides. Such fusion polypeptides may possess modified biological, biochemical, and/or immunological properties in comparison to wild-type α_d. Analog polypeptides including additional amino acid (*e.g.*, lysine or cysteine) residues that facilitate multimer formation are contemplated.

Also comprehended by the present invention are polypeptides and other non-peptide molecules which specifically bind to α_d. Preferred binding molecules include antibodies (*e.g.*, monoclonal and polyclonal antibodies), counterreceptors (*e.g.*, membrane-associated and soluble forms) and other ligands (*e.g.*, naturally occurring or synthetic molecules), including those which competitively bind α_d in the presence of α_d monoclonal antibodies and/or specific counterreceptors. Binding molecules are useful for purification of α_d polypeptides and identifying cell types which express α_d. Binding molecules are also useful for modulating (*i.e.*, inhibiting, blocking or stimulating) of *in vivo* binding and/or signal transduction activities of α_d.

Assays to identify α_d binding molecules are also provided, including *in vitro* assays such as immobilized ligand binding assays, solution binding assays, and scintillation proximity assays, as well as cell based assays such as di-hybrid screening assays, split hybrid screening assays, and the like. Cell based assays provide for a phenotypic change in a host cell as a result of specific binding interaction or disruption of a specific binding interaction, thereby permitting indirect quantitation or measurement of some specific binding interaction.

In vitro assays for identifying antibodies or other compounds that modulate the activity of α_d may involve, for example, immobilizing α_d or a natural ligand to which α_d binds, detectably labelling the nonimmobilized binding partner, incubating the binding partners together and determining the effect of a test compound on the amount of label bound wherein a reduction in the label bound in the presence of the test compound compared to the amount of label bound in the absence of the test compound indicates that the test agent is an inhibitor of α_d binding.

Another type of *in vitro* assay for identifying compounds that modulate the

interaction between α_d and a ligand involves immobilizing α_d or a fragment thereof on a solid support coated (or impregnated with) a fluorescent agent, labeling the ligand with a compound capable of exciting the fluorescent agent, contacting the immobilized α_d with the labeled ligand in the presence and absence of a putative modulator compound, detecting light emission by the fluorescent agent, and identifying modulating compounds as those compounds that affect the emission of light by the fluorescent agent in comparison to the emission of light by the fluorescent agent in the absence of a modulating compound. Alternatively, the α_d ligand may be immobilized and α_d may be labeled in the assay.

A cell based assay method contemplated by the invention for identifying compounds that modulate the interaction between α_d and a ligand involves transforming or transfecting appropriate host cells with a DNA construct comprising a reporter gene under the control of a promoter regulated by a transcription factor having a DNA-binding domain and an activating domain, expressing in the host cells a first hybrid DNA sequence encoding a first fusion of part or all of α_d and either the DNA binding domain or the activating domain of the transcription factor, expressing in the host cells a second hybrid DNA sequence encoding part or all of the ligand and the DNA binding domain or activating domain of the transcription factor which is not incorporated in the first fusion, evaluating the effect of a putative modulating compound on the interaction between α_d and the ligand by detecting binding of the ligand to α_d in a particular host cell by measuring the production of reporter gene product in the host cell in the presence or absence of the putative modulator, and identifying modulating compounds as those compounds altering production of the reported gene product in comparison to production of the reporter gene product in the absence of the modulating compound. Presently preferred for use in the assay are the *lexA* promoter, the *lexA* DNA binding domain, the ~~GAL4~~ transactivation domain, the *lacZ* reporter gene, and a yeast host cell.

A modified version of the foregoing assay may be used in isolating a polynucleotide encoding a protein that binds to α_d by transforming or transfecting appropriate host cells with a DNA construct comprising a reporter gene under the control of a promoter regulated by a transcription factor having a DNA-binding domain and an activating domain, expressing in the host cells a first hybrid DNA sequence encoding a

first fusion of part or all of α_d and either the DNA binding domain or the activating domain of the transcription factor, expressing in the host cells a library of second hybrid DNA sequences encoding second fusions of part or all of putative α_d binding proteins and the DNA binding domain or activating domain of the transcription factor which is not incorporated in the first fusion, detecting binding of an α_d binding protein to α_d in a particular host cell by detecting the production of reporter gene product in the host cell, and isolating second hybrid DNA sequences encoding α_d binding protein from the particular host cell.

In a preferred embodiment utilizing the split hybrid assay, the invention provides a method to identify an inhibitor of binding between an α_d protein or fragment thereof and an α_d binding protein or binding fragment thereof comprising the steps of: (a) transforming or transfecting a host cell with a first DNA expression construct comprising a first selectable marker gene encoding a first selectable marker protein and a repressor gene encoding a repressor protein, said repressor gene under transcriptional control of a promoter; (b) transforming or transfecting said host cell with a second DNA expression construct comprising a second selectable marker gene encoding a second selectable marker protein and a third selectable marker gene encoding a third selectable marker protein, said third selectable marker gene under transcriptional control of an operator, said operator specifically acted upon by said repressor protein such that interaction of said repressor protein with said operator decreases expression of said third selectable marker protein; (c) transforming or transfecting said host cell with a third DNA expression construct comprising a fourth selectable marker gene encoding a fourth selectable marker protein and an α_d fusion protein gene encoding an α_d protein or fragment thereof in frame with either a DNA binding domain of a transcriptional activation protein or a transactivating domain of said transcriptional activation protein; (d) transforming or transfecting said host cell with a fourth DNA expression construct comprising a fifth selectable marker gene encoding a fifth selectable marker protein and a second fusion protein gene encoding an α_d binding protein or binding fragment thereof in frame with either the DNA binding domain of said transcriptional activation protein or the transactivating domain of said transcriptional activation protein, whichever is not included in first fusion protein gene; (e) growing said host cell under conditions which permit

expression of said α_d protein or fragment thereof and said α_d binding protein or fragment thereof such that said α_d protein or fragment thereof and α_d binding protein or binding fragment thereof interact bringing into proximity said DNA binding domain and said transactivating domain reconstituting said transcriptional activating protein; said transcriptional activating protein acting on said promoter to increase expression of said repressor protein; said repressor protein interacting with said operator such that said third selectable marker protein is not expressed; (f) detecting absence of expression of said selectable gene; (g) growing said host cell in the presence of a test inhibitor of binding between said α_d protein or fragment thereof and said α_d binding protein or fragment thereof; and (h) comparing expression of said selectable marker protein in the presence and absence of said test inhibitor wherein decreased expression of said selectable marker protein is indicative of an ability of the test inhibitor to inhibit binding between said α_d protein or fragment thereof and said α_d binding protein or binding fragment thereof such that said transcriptional activating protein is not reconstituted, expression of said repressor protein is not increased, and said operator increases expression of said selectable marker protein.

The invention comprehends host cells wherein the various genes and regulatory sequences are encoded on a single DNA molecule as well as host cells wherein one or more of the repressor gene, the selectable marker gene, the α_d fusion protein gene, and the α_d binding protein gene are encoded on distinct DNA expression constructs. In a preferred embodiment, the host cells are transformed or transfected with DNA encoding the repressor gene, the selectable marker gene, the α_d fusion protein gene, and the $_d$ fusion binding protein gene, each encoded on a distinct expression construct. Regardless of the number of DNA expression constructs introduced, each transformed or transfected DNA expression construct further comprises a selectable marker gene sequence, the expression of which is used to confirm that transfection or transformation was, in fact, accomplished. Selectable marker genes encoded on individually transformed or transfected DNA expression constructs are distinguishable from the selectable marker under transcriptional regulation of the *tet* operator in that expression of the selectable marker gene regulated by the *tet* operator is central to the preferred embodiment; *i.e.*, regulated expression of the selectable marker gene by the *tet* operator provides a measurable phenotypic change in the

host cell that is used to identify a binding protein inhibitor. Selectable marker genes encoded on individually transformed or transfected DNA expression constructs are provided as determinants of successful transfection or transformation of the individual DNA expression constructs. Preferred host cells of the invention include transformed *S. cerevisiae* strains designated YI596 and YI584 which were deposited August 13, 1996 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, and assigned Accession Numbers ATCC 74384 and ATCC 74385, respectively.

The host cells of the invention include any cell type capable of expressing the α_d and α_d binding proteins required as described above and which are capable of being transformed or transfected with functional promoter and operator sequences which regulate expression of the heterologous proteins also as described. In a preferred embodiment, the host cells are of either mammal, insect or yeast origin. Presently, the most preferred host cell is a yeast cell. The preferred yeast cells of the invention can be selected from various strains, including the *S. cerevisiae* yeast transformants described in Table 1. Alternative yeast specimens include *S.pombe, K.lactis, P.pastoris, S.carlsbergensis* and *C.albicans*. Preferred mammalian host cells of the invention include Chinese hamster ovary (CHO), COS, HeLa, 3T3, CV1, LTK, 293T3, Rat1, PC12 or any other transfectable cell line of human or rodent origin. Preferred insect cell lines include SF9 cells.

In a preferred embodiment, the selectable marker gene is regulated by an operator and encodes an enzyme in a pathway for synthesis of a nutritional requirement for said host cell such that expression of said selectable marker protein is required for growth of said host cell on media lacking said nutritional requirement. Thus, as in a preferred embodiment where a repressor protein interacts with the operator, transcription of the selectable marker gene is down-regulated and the host cells are identified by an inability to grow on media lacking the nutritional requirement and an ability to grow on media containing the nutritional requirement. In a most preferred embodiment, the selectable marker gene encodes the HIS3 protein, and host cells transformed or transfected with a HIS3-encoding DNA expression construct are selected following growth on media in the presence and absence of histidine. The invention, however,

comprehends any of a number of alternative selectable marker genes regulated by an operator. Gene alternatives include, for example, *URA3, LEU2, LYS2* or those encoding any of the multitude of enzymes required in various pathways for production of a nutritional requirement which can be definitively excluded from the media of growth. In addition, conventional reporter genes such as chloramphenicol acetyltransferase (CAT), firefly luciferase, β-galactosidase (β-gal), secreted alkaline phosphatase (SEAP), green fluorescent protein (GFP), human growth hormone (hGH), β-glucuronidase, neomycin, hygromycin, thymidine kinase (TK) and the like may be utilized in the invention.

In the preferred embodiment, the host cells include a repressor protein gene encoding the tetracycline resistance protein which acts on the *tet* operator to decrease expression of the selectable marker gene. The invention, however, also encompasses alternatives to the *tet* repressor and operator, for example, *E.coli trp* repressor and operator, *his* repressor and operator, and *lac* operon repressor and operator.

The DNA binding domain and transactivating domain components of the fusion proteins may be derived from the same transcription factor or from different transcription factors as long as bringing the two domains into proximity through binding between α_d and the α_d binding protein permits formation of a functional transcriptional activating protein that increases expression of the repressor protein with high efficiency. A high efficiency transcriptional activating protein is defined as having both a DNA binding domain exhibiting high affinity binding for the recognized promoter sequence and a transactivating domain having high affinity binding for transcriptional machinery proteins required to express repressor gene mRNA. The DNA binding domain component of a fusion protein of the invention can be derived from any of a number of different proteins including, for example, LexA or Gal4. Similarly, the transactivating component of the invention's fusion proteins can be derived from a number of different transcriptional activating proteins, including for example, Gal4 or VP16.

The promoter sequence of the invention which regulates transcription of the repressor protein can be any sequence capable of driving transcription in the chosen host cell. The promoter may be a DNA sequence specifically recognized by the chosen DNA binding domain of the invention, or any other DNA sequence with which the DNA binding domain of the fusion protein is capable of high affinity interaction. In a preferred

embodiment of the invention, the promoter sequence of the invention is either a HIS3 or alcohol dehydrogenase (ADH) promoter. In a presently most preferred embodiment, the ADH promotor is employed in the invention. The invention, however, encompasses numerous alternative promoters, including, for example, those derived from genes encoding HIS3, ADH, URA3, LEU2 and the like.

The methods of the invention encompass any and all of the variations in host cells as described above. In particular, the invention encompasses a method wherein: the host cell is a yeast cell; the selectable marker gene encodes HIS3; transcription of the selectable marker gene is regulated by the *tet* operator; the repressor protein gene encodes the tetracycline resistance protein; transcription of the tetracycline resistance protein is regulated by the HIS3 promoter; the DNA binding domain is derived from LexA; and the transactivating domain is derived from VP16. In another embodiment, the invention encompasses a method wherein: the host cell is a yeast cell; the selectable marker gene encodes HIS3; transcription of the selectable marker gene is regulated by the *tet* operator; the repressor protein gene encodes the tetracycline resistance protein; transcription of the tetracycline resistance protein is regulated by the alcohol dehydrogenase promoter; the DNA binding domain is derived from LexA; and the transactivating domain is derived from VP16.

In alternative embodiments of the invention wherein the host cell is a mammalian cell, variations include the use of mammalian DNA expression constructs to encode the α_d and α_d binding fusion genes, the repressor gene, and the selectable marker gene, and use of selectable marker genes encoding antibiotic or drug resistance markers (i.e., neomycin, hygromycin, thymidine kinase).

There are at least three different types of libraries used for the identification of small molecule modulators. These include: (1) chemical libraries, (2) natural product libraries, and (3) combinatorial libraries comprised of random peptides, oligonucleotides or organic molecules.

Chemical libraries consist of structural analogs of known compounds or compounds that are identified as "hits" via natural product screening. Natural product libraries are collections of microorganisms, animals, plants, or marine organisms which are used to create mixtures for screening by: (1) fermentation and extraction of broths from

soil, plant or marine microorganisms or (2) extraction of plants or marine organisms. Combinatorial libraries are composed of large numbers of peptides, oligonucleotides or organic compounds as a mixture. They are relatively easy to prepare by traditional automated synthesis methods, PCR, cloning or proprietary synthetic methods. Of particular interest are peptide and oligonucleotide combinatorial libraries. Still other libraries of interest include peptide, protein, peptidomimetic, multiparallel synthetic collection, recombinatorial, and polypeptide libraries.

Hybridoma cell lines which produce antibodies specific for α_d are also comprehended by the invention. Techniques for producing hybridomas which secrete monoclonal antibodies are well known in the art. Hybridoma cell lines may be generated after immunizing an animal with purified α_d, variants of α_d or cells which express α_d or a variant thereof on the extracellular membrane surface. Immunogen cell types include cells which express α_d *in vivo*, or transfected prokaryotic or eukaryotic cell lines which normally do not normally express α_d *in vivo*. Presently preferred antibodies of the invention are secreted by hybridomas designated 169A, 169B, 170D, 170F, 170E, 170X, 170H, 188A, 188B, 188C, 188E, 188F, 188G, 188I, 188J, 188K, 188L, 188M, 188N, 188P, 188R, 188T, 195A, 195C, 195D, 195E, 195H, 197A-1, 197A-2, 197A-3, 197A-4, 199A, 199H, 199M, 205A, 205C, 205E, 212A, 212D, 217G, 217H, 217I, 217K, 217L, 217M, 226A, 226B, 226C, 226D, 226E, 226F, 226G, 226H, 226I, 236A, 236B, 236C, 236F, 236G, 236H, 236I, 236K, 237L, 236M, 240F, 240G, 240H, and 236L.

The value of the information contributed through the disclosure of the DNA and amino acid sequences of α_d is manifest. In one series of examples, the disclosed α_d CDNA sequence makes possible the isolation of the human α_d genomic DNA sequence, including transcriptional control elements for the genomic sequence. Identification of α_d allelic variants and heterologous species (*e.g.*, rat or mouse) DNAs is also comprehended. Isolation of the human α_d genomic DNA and heterologous species DNAs can be accomplished by standard DNA/DNA hybridization techniques, under appropriately stringent conditions, using all or part of the α_d cDNA sequence as a probe to screen an appropriate library. Alternatively, polymerase chain reaction (PCR) using oligonucleotide primers that are designed based on the known cDNA sequence can be used to amplify and identify genomic α_d DNA sequences. Synthetic DNAs encoding the α_d polypeptide,

including fragments and other variants thereof, may be produced by conventional synthesis methods.

DNA sequence information of the invention also makes possible the development, by homologous recombination or "knockout" strategies [see, *e.g.*, Kapecchi, *Science 244*:1288-1292 (1989)], to produce rodents that fail to express a functional α_d polypeptide or that express a variant α_d polypeptide. Such rodents are useful as models for studying the activities of α_d and α_d modulators *in vivo*.

DNA and amino acid sequences of the invention also make possible the analysis of α_d epitopes which actively participate in counterreceptor binding as well as epitopes which may regulate, rather than actively participate in, binding. Identification of epitopes which may participate in transmembrane signal transduction is also comprehended by the invention.

DNA of the invention is also useful for the detection of cell types which express α_d polypeptide. Standard DNA/RNA hybridization techniques which utilize α_d DNA to detect α_d RNA may be used to determine the constitutive level of α_d transcription within a cell, as well as changes in the level of transcription in response to internal or external agents. Identification of agents which modify transcription and/or translation of α_d can, in turn, be assessed for potential therapeutic or prophylactic value. DNA of the invention also makes possible *in situ* hybridization of α_d DNA to cellular RNA to determine the cellular localization of α_d specific messages within complex cell populations and tissues.

DNA of the invention is also useful for identification of non-human polynucleotide sequences which display homology to human α_d sequences. Possession of non-human α_d DNA sequences permits development of animal models (including, for example, transgenic models) of the human system.

As another aspect of the invention, monoclonal or polyclonal antibodies specific for α_d may be employed in immunohistochemical analysis to localize α_d to subcellular compartments or individual cells within tissues. Immunohistochemical analyses of this type are particularly useful when used in combination with *in situ* hybridization to localize both α_d mRNA and polypeptide products of the α_d gene.

Identification of cell types which express α_d may have significant

ramifications for development of therapeutic and prophylactic agents. It is anticipated that the products of the invention related to α_d can be employed in the treatment of diseases wherein macrophages are an essential element of the disease process. Animal models for many pathological conditions associated with macrophage activity have been described in the art. For example, in mice, macrophage recruitment to sites of both chronic and acute inflammation is reported by Jutila, *et al., J.Leukocyte Biol. 54*:30-39 (1993). In rats, Adams, *et al.*, [*Transplantation 53*:1115-1119(1992) and *Transplantation 56*:794-799 (1993)] describe a model for graft arteriosclerosis following heterotropic abdominal cardiac allograft transplantation. Rosenfeld, *et al.*, [*Arteriosclerosis 7*:9-23 (1987) and *Arteriosclerosis 7*:24-34 (1987)] describe induced atherosclerosis in rabbits fed a cholesterol supplemented diet. Hanenberg, *et al.*, [*Diabetologia 32*:126-134 (1989)] report the spontaneous development of insulin-dependent diabetes in BB rats. Yamada *et al.*, [*Gastroenterology 104*:759-771 (1993)] describe an induced inflammatory bowel disease, chronic granulomatous colitis, in rats following injections of streptococcal peptidoglycan-polysaccharide polymers. Cromartie, *et al.*, [*J.Exp.Med. 146*:1585-1602 (1977)] and Schwab, *et al.*, [*Infection and Immunity 59*:4436-4442 (1991)] report that injection of streptococcal cell wall protein into rats results in an arthritic condition characterized by inflammation of peripheral joints and subsequent joint destruction. Finally, Huitinga, *et al.*, [*Eur.J.Immunol 23*:709-715 (1993) describe experimental allergic encephalomyelitis, a model for multiple sclerosis, in Lewis rats. In each of these models, α_d antibodies, other α_d binding proteins, or soluble forms of α_d are utilized to attenuate the disease state, presumably through inactivation of macrophage activity.

Pharmaceutical compositions for treatment of these and other disease states are provided by the invention. Pharmaceutical compositions are designed for the purpose of inhibiting interaction between α_d and its ligand(s) and include various soluble and membrane-associated forms of α_d (comprising the entire α_d polypeptide, or fragments thereof which actively participate in α_d binding), soluble and membrane-associated forms of α_d binding proteins (including antibodies, ligands, and the like), intracellular or extracellular modulators of α_d binding activity, and/or modulators of α_d and/or α_d-ligand polypeptide expression, including modulators of transcription, translation, post-translational processing and/or intracellular transport.

The invention also comprehends methods for treatment of disease states in which α_d binding, or localized accumulation of cells which express α_d, is implicated, wherein a patient suffering from said disease state is provided an amount of a pharmaceutical composition of the invention sufficient to modulate levels of α_d binding or to modulate accumulation of cell types which express α_d. The method of treatment of the invention is applicable to disease states such as, but not limited to, Type I diabetes, atherosclerosis, multiple sclerosis, asthma, psoriasis, lung inflammation, acute respiratory distress syndrome and rheumatoid arthritis.

The invention also provides methods for inhibiting macrophage infiltration at the site of a central nervous system injury comprising the step of administering to an individual an effective amount of an anti-α_d monoclonal antibody. In one aspect, the methods comprise use of an anti-α_d monoclonal antibody that blocks binding between α_d and a binding partner. In one embodiment, the binding partner is VCAM-1. In a preferred embodiment, the anti-α_d monoclonal antibody is selected from the group consisting of the monoclonal antibody secreted by hybridoma 226H and the monoclonal antibody secreted by hybridoma 236L. In a most preferred embodiment, methods of the invention are for a central nervous system injury which is a spinal cord injury.

The invention further provides methods for reducing inflammation at the site of a central nervous system injury comprising the step of administering to an individual an effective amount of an anti-α_d monoclonal antibody. In one aspect, the methods comprise use of an anti-α_d monoclonal antibody that blocks binding between α_d and a binding partner. In one embodiment, the binding partner is VCAM-1. In a preferred embodiment, the anti-α_d monoclonal antibody is selected from the group consisting of the monoclonal antibody secreted by hybridoma 226H and the monoclonal antibody secreted by hybridoma 236L. In a most preferred embodiment, methods of the invention are for a central nervous system injury which is a spinal cord injury.

Hybridomas 226H and 236L were received on November 11, 1998 by the American Type Culture Collection, 10801 University Boulevard, Masassas, VA 20110-2209 under terms of the Budapest Treaty and assigned Accession Nos: _____ and _____, respectively.

The invention also provides methods for modulating TNFα release from

macrophage or splenic phagocytes comprising the step of contacting said phagocytes with an affective amount of an immunospecific α_d monoclonal antibody. In a preferred aspect, the method methods of the invention comprise an anti-monoclonal antibody that inhibits TNFα release. In a preferred embodiment, the methods of the invention comprise use of an immunospecific anti-α_d monoclonal antibody that is selected from the group consisting of the monoclonal antibody secreted by hybridoma 205C and the monoclonal antibody secreted by hybridoma 205E.

Methods of the invention are contemplated wherein useful antibodies include fragments of anti-α_d monoclonal antibodies, including for example, Fab or $F(ab')_2$ fragments. Methods utilizing modified antibodies are also embraced by the invention. Modified antibodies include, for example, single chain antibodies, chimeric antibodies, and CDR-grafted antibodies, including compounds which include CDR sequences which specifically recognize a polypeptide of the invention, as well as humanized antibodies. Methods comprising use of human antibodies are also contemplated. Techniques for identifying and isolating human antibodies are disclosed *infra*.

The invention also provides methods for inhibiting macrophage infiltration at the site of a central nervous system injury comprising the step of administering to an individual an effective amount of a small molecule that inhibits α_d binding. In particular, the methods of the invention comprising a central nervous system injury which is a spinal cord injury. Small molecules specific for α_d binding are identified and isolated from libraries as discussed above.

The invention further provides methods for reducing inflammation at the site of a central nervous system injury comprising the step of administering to an individual an effective amount of a small molecule that inhibits α_d binding. In particular, the methods of the invention comprising a central nervous system injury which is a spinal cord injury. Small molecules specific for α_d binding are identified and isolated from libraries as discussed above.

The invention further embraces methods to detect and diagnose Crohn's disease comprising the steps of obtaining tissue samples from a patient; staining the sample with anti-α_d monoclonal antibodies, and comparing the staining pattern to that on tissue obtained from a known normal donor. In instances wherein staining differences between

the two tissue samples can be detected, the patient can be further tested for possible Crohn's disease.

The invention also contemplates use of α_d as a target for removal of pathogenic cell populations expressing α_d on the cell surface. In one aspect, the hypervariable region of an α_d monoclonal antibody is cloned and expressed in the context of a complement-fixing human isotype. Cloning the hypervariable region in this manner will provide a binding partner for α_d, which unpon binding *in vivo*, will lead to complement binding and subsequent cell death. Alternatively, the anti-α_d monoclonal antibody is conjugated to a cytotoxic compound and binding of the antibody to α_d on the pathogenic cell type leads to cell death.

Brief Description of the Drawing

Numerous other aspects and advantages of the present invention will be apparent upon consideration of the following description thereof, reference being made to the drawing wherein:

Figure 1A through 1D comprises an alignment of the human amino acid sequences of CD11b (SEQ ID NO: 3), CD11c (SEQ ID NO: 4) and α_d (SEQ ID NO: 2).

Detailed Description of the Invention

The present invention is illustrated by the following examples relating to the isolation of a cDNA clone encoding α_d from a human spleen cDNA library. More particularly, Example 1 illustrates the use of anti-canine α_{TM1} antibody in an attempt to detect a homologous human protein. Example 2 details purification of canine α_{TM1} and N-terminal sequencing of the polypeptide to design oligonucleotide primers for PCR amplification of the canine α_{TM1} gene. Example 3 addresses large scale purification of canine α_{TM1} for internal sequencing in order to design additional PCR primers. Example 4 describes use of the PCR and internal sequence primers to amplify a fragment of the canine α_{TM1} gene. Example 5 addresses cloning of the human α_d-encoding cDNA sequence. Example 6 describes Northern blot hybridization analysis of human tissues and cells for expression of α_d mRNA. Example 7 details the construction of human α_d expression plasmids and transfection of COS cells with the resulting plasmids. Example

8 addresses ELISA analysis of α_d expression in transfected COS cells. Example 9 describes FACS analysis of COS cells transfected with human α_d expression plasmids. Example 10 addresses immunoprecipitation of CD18 in association with α_d in co-transfected COS cells. Example 11 relates to stable transfection of α_d expression constructs in Chinese hamster ovary cells. Example 12 addresses CD18-dependent binding of α_d to the intercellular adhesion molecule ICAM-R, an ICAM-R mutant protein, and complement fact iC3b. Example 13 describes scintillation proximity screening assays to identify inhibitors or enhancers (*i.e.*, modulators) of α_d ligand/anti-ligand binding interactions. Example 14 addresses construction of expression plasmids which encode soluble forms of α_d, and binding analyses of the expression products. Example 15 relates to production of α_d-specific polyclonal sera and monoclonal antibodies. Example 16 describes flow cytometry analysis using α_d monoclonal antibodies. Example 17 addresses expression of α_d on human monocytes. Example 18 describes analysis of α_d tissue distribution, expression of α_d on peripheral blood leukocytes, expression in inflammatory and non-inflammatory synovium using anti-α_d polyclonal serum, expression in disease lung and liver, human bone marrow, and PBMC from breast cancer patients. Example 19 addresses upregulation of α_d expression *in vitro* and *in vivo*. Example 20 describes isolation of rat cDNA sequences which show homology to human α_d gene sequences. Example 21 addresses tissue specific expression of rat α_d mRNA. Example 22 relates to construction of full length rat α_d expression plasmids, rat α_d I domain expression plasmids, including I domain/IgG fusion proteins, production of monoclonal antibodies to full length and I domain fusion proteins, and production of polyclonal antisera to rat α_d I domain sequences fused to human IgG4. Example 23 describes specificity of monoclonal antibody 199M. Example 24 presents results from a T cell proliferation assay using rat α_d expressing macrophages. Example 25 describes immunoprecipitation of rat α_d from bone marrow. Example 26 describes rat α_d expression in various animal models. Example 27 relates to an assay for inhibition of NK-tumor cell-induced target cell lysis using α_d monoclonal antibodies. Example 28 addresses isolation of mouse cDNA sequences which show homology to human α_d gene sequences. Example 29 describes isolation of additional mouse α_d cDNA clones used to confirm sequence analysis. Example 30 relates to *in situ* hybridization analysis of various mouse tissues to determine tissue and cell

specific expression of the putative mouse homolog to human α_d. Example 31 describes generation of expression constructs which encode the putative mouse homolog of human α_d. Example 32 addresses design of a "knock-out" mouse wherein the gene encoding the putative mouse homolog of human α_d is disrupted. Example 33 describes isolation of rabbit cDNA clones which show homology to human α_d encoding sequences. Example 34 describes isolation of monkey α_d. Example 35 relates to characterization of the antigen recognized by monoclonal antibody 217L. Example 36 describes animal models of human disease states wherein modulation of α_d is assayed for therapeutic capabilities. Example 37 describes expression of α_d in animal model disease states. 38 addresses the role of α_d in spinal cord injury. Example 39 describes α_d expression in Crohn's disease. Example 40 relates to TNFα release from rat spleen cells that express α_d. Example 41 described methods to modulate TNFα release from spleen cells using α_d monoclonal antibodies. Example 42 characterizes α_d expression on eosinophils. Example 43 relates to further characterization of α_d binding to VCAM-1. Example 44 described use of α_d as a target for removal of pathogenic cell populations.

Example 1

Attempt to Detect a Human Homolog of Canine α_{TM1}

The monoclonal antibody Ca11.8H2 [Moore, et al., *supra*] specific for canine α_{TM1} was tested for cross-reactivity on human peripheral blood leukocytes in an attempt to identify a human homolog of canine α_{TM1}. Cell preparations (typically 1 x 10^6 cells) were incubated with undiluted hybridoma supernatant or a purified mouse IgG-negative control antibody (10 µg/ml) on ice in the presence of 0.1% sodium azide. Monoclonal antibody binding was detected by subsequent incubation with FITC-conjugated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) at 6 µg/ml. Stained cells were fixed with 2% w/v paraformaldehyde in phosphate buffered saline (PBS) and were analyzed with a Facstar Plus fluorescence-activated cell sorter (Becton Dickinson, Mountain View, CA). Typically, 10,000 cells were analyzed using logarithmic amplification for fluorescence intensity.

The results indicated that Ca11.8H2 did not cross-react with surface proteins expressed on human peripheral blood leukocytes, while the control cells, neoplastic canine peripheral blood lymphocytes, were essentially all positive for α_{TM1}.

Because the monoclonal antibody Ca11.8H2 specific for the canine α subunit did not cross react with a human homolog, isolation of canine α_{TM1} DNA was deemed a necessary prerequisite to isolate a counterpart human gene if one existed.

Example 2
Affinity Purification Of Canine α_{TM1} For N-Terminal Sequencing

Canine α_{TM1} was affinity purified in order to determine N-terminal amino acid sequences for oligonucleotide probe/primer design. Briefly, anti-α_{TM1} monoclonal antibody Ca11.8H2 was coupled to Affigel® 10 chromatographic resin (BioRad, Hercules, CA) and protein was isolated by specific antibody-protein interaction. Antibody was conjugated to the resin, according to the BioRad suggested protocol, at a concentration of approximately 5 mg antibody per ml of resin. Following the conjugation reaction, excess antibody was removed and the resin blocked with three volumes of 0.1 M ethanolamine. The resin was then washed with thirty column volumes of phosphate buffered saline (PBS).

Twenty-five grams of a single dog spleen were homogenized in 250 ml of buffer containing 0.32 M sucrose in 25 mM Tris-HCl, Ph 8.0, with protease inhibitors. Nuclei and cellular debris were pelleted with centrifugation at 1000 g for 15 minutes. Membranes were pelleted from the supernatant with centrifugation at 100,000 g for 30 minutes. The membrane pellet was resuspended in 200 ml lysis buffer (50 mM NaCl, 50 mM borate, pH 8.0, with 2% NP-40) and incubated for 1 hour on ice. Insoluble material was then pelleted by centrifugation at 100,000 g for 60 minutes. Ten milliliters of the cleared lysate were transferred to a 15 ml polypropylene tube with 0.5 ml Ca11.8H2-conjugated Affigel® 10 resin described above. The tube was incubated overnight at 4°C with rotation and the resin subsequently washed with 50 column volumes D-PBS. The resin was then transferred to a microfuge tube and boiled for ten minutes in 1 ml Laemmli (non-reducing) sample buffer containing 0.1 M Tris-HCl, pH 6.8, 2% SDS, 20% glycerol and 0.002% bromophenol blue. The resin was pelleted by centrifugation and discarded; the supernatant was treated with 1/15 volume β-mercaptoethanol (Sigma, St. Louis, MO) and run on a 7% polyacrylamide gel. The separated proteins were transferred to Immobilon PVDF membrane (Millipore, Bedford, MA) as follows.

The gels were washed once in deionized, Millipore®-filtered water and

equilibrated for 15-45 minutes in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) transfer buffer, pH 10.5, with 10% methanol. Immobilon membranes were moistened with methanol, rinsed with filtered water, and equilibrated for 15-30 minutes in CAPS transfer buffer. The initial transfer was carried out using a Biorad transfer apparatus at 70 volts for 3 hours. The Immobilon membrane was removed after transfer and stained in filtered 0.1% R250 Coomassie stain for 10 minutes. Membranes were destained in 50% methanol/10% acetic acid three times, ten minutes each time. After destaining, the membranes were washed in filtered water and air-dried.

Protein bands of approximately 150 kD, 95 kD, 50 kD and 30 kD were detected. Presumably the 50 kD and 30 kD bands resulted from antibody contamination. N-terminal sequencing was then attempted on both the 150 kD and 95 kD bands, but the 95 kD protein was blocked, preventing sequencing. The protein band of 150 kD was excised from the membrane and directly sequenced with an Applied Biosystems (Foster City, CA) Model 473A protein sequencer according to the manufacturer's instructions. The resulting amino acid sequence is set in SEQ ID NO: 5 using single letter amino acid designations.

FNLDVEEPMVFQ———— (SEQ ID NO: 5)

The identified sequence included the FNLD sequence characteristic of α subunits of the integrin family [Tamura, *et al.*, *J.Cell.Biol. 111*:1593-1604 (1990)].

Primer Design and Attempt to Amplify Canine α_{TM1} Sequences

From the N-terminal sequence information, three oligonucleotide probes were designed for hybridization: a) "Tommer," a fully degenerate oligonucleotide; b) "Patmer," a partially degenerate oligonucleotide; and c) "Guessmer," a nondegenerate oligonucleotide based on mammalian codon usage. These probes are set out below as SEQ ID NOS: 6, 7 and 8, respectively. Nucleic acid symbols are in accordance with 37 C.F.R. §1.882 for these and all other nucleotide sequences herein.

5′-TTYAAYYTGGAYGTNGARGARCCNATGGTNTTYCA-3′

(SEQ ID NO: 6)

5′-TTCAACCTGGACGTGGAGGAGCCCATGGTGTTCCAA-3′

(SEQ ID NO: 7)

5′-TTCAACCTGGACGTNGAASANCCCATGGTCTTCCAA-3′

(SEQ ID NO: 8)

Based on sequencing data, no relevant clones were detected using these oligonucleotides in several low stringency hybridizations to a canine spleen/peripheral blood macrophage cDNA library cloned into λZAP® (Stratagene, La Jolla, CA).

Four other oligonucleotide primers, designated 5′Deg, 5′Spec, 3′Deg and 3′Spec (as set out in SEQ ID NOS: 9, 10, 11 and 12, respectively, wherein Deg indicates degenerate and Spec indicates non-degenerate) were subsequently designed based on the deduced N-terminal sequence for attempts to amplify canine α_{TM1} sequences by PCR from phage library DNA purified from plate lysates of the Stratagene library described above.

5′-TTYAAYYTNGAYGTNGARGARCC-3′ (SEQ ID NO: 9)

5′-TTYAAYYTGGACGTNGAAGA-3′ (SEQ ID NO: 10)

5′-TGRAANACCATNGGYTC-3′ (SEQ ID NO: 11)

5′-TTGGAAGACCATNGGYTC-3′ (SEQ ID NO: 12)

The α_{TM1} oligonucleotide primers were paired with T3 or T7 vector primers, as set out in SEQ ID NOS: 13 and 14, respectively, which hybridize to sequences flanking the polylinker region in the Bluescript® phagemid found in λZAP®.

5′-ATTAACCCTCACTAAAG-3′ (SEQ ID NO: 13)

5′-AATACGACTCACTATAG-3′ (SEQ ID NO: 14)

The PCR amplification was carried out in *Taq* buffer (Boehringer Mannheim, Indianapolis, IN) containing magnesium with 150 ng of library DNA, 1 μg of each primer, 200 μM

dNTPs and 2.5 units *Taq* polymerase (Boehringer Mannheim) and the products were separated by electrophoresis on a 1% agarose gel in Tris-Acetate-EDTA (TAE) buffer with 0.25 μg/ml ethidium bromide. DNA was transferred to a Hybond® (Amersham, Arlington Heights, IL) membrane by wicking overnight in 10X SSPE. After transfer, the immobilized DNA was denatured with 0.5 M NaOH with 0.6 M NaCl, neutralized with 1.0 M Tris-HCl, pH 8.0, in 1.5 M NaCl, and washed with 2X SSPE before UV crosslinking with a Stratalinker (Stratagene) crosslinking apparatus. The membrane was incubated in prehybridization buffer (5X SSPE, 4X Denhardts, 0.8% SDS, 30% formamide) for 2 hr at 50°C with agitation.

Oligonucleotide probes 5′Deg, 5′Spec, 3′Deg and 3′Spec (SEQ ID NOS: 9, 10, 11 and 12, respectively) were labeled using a Boehringer Mannheim kinase buffer with 100-300 μCi γP^{32}-dATP and 1-3 units of polynucleotide kinase for 1-3 hr at 37°C. Unincorporated label was removed with Sephadex® G-25 fine (Pharmacia, Piscataway, NJ) chromatography using 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (TE) buffer and the flow-through added directly to the prehybridization solution. Membranes were probed for 16 hr at 42°C with agitation and washed repeatedly, with a final stringency wash of 1X SSPE/0.1% SDS at 50° for 15 min. The blot was then exposed to Kodak X-Omat AR film for 1-4 hours at -80°C.

The oligonucleotides 5′Deg, 5′Spec, 3′Deg and 3′Spec only hybridized to PCR products from the reactions in which they were used as primers and failed to hybridize as expected to PCR products from the reactions in which they were not used as primers. Thus, it was concluded that none of the PCR products were specific for α_{TM1} because no product hybridized with all of the appropriate probes.

Example 3

Large Scale Affinity Purification Of Canine α_{TM1} For Internal Sequencing

In order to provide additional amino acid sequence for primer design, canine α_{TM1} was purified for internal sequencing. Three sections of frozen spleen (approximately 50 g each) and frozen cells from two partial spleens from adult dogs were used to generate protein for internal sequencing. Fifty grams of spleen were homogenized in 200-300 ml borate buffer with a Waring blender. The homogenized material was diluted with 1 volume of buffer containing 4% NP-40, and the mixture then gently

agitated for at least one hour. The resulting lysate was cleared of large debris by centrifugation at 2000 g for 20 min, and then filtered through either a Corning (Corning, NY) prefilter or a Corning 0.8 micron filter. The lysate was further clarified by filtration through the Corning 0.4 micron filter system.

Splenic lysate and the antibody-conjugated Affigel® 10 resin described in Example 2 were combined at a 150:1 volume ratio in 100 ml aliquots and incubated overnight at 4°C with rocking. The lysate was removed after centrifugation at 1000 g for 5 minutes, combined with more antibody-conjugated Affigel® 10 resin and incubated overnight as above. The absorbed resin aliquots were then combined and washed with 50 volumes D-PBS/0.1% Tween® 20 and the resin transferred to a 50 ml Biorad column. Adsorbed protein was eluted from the resin with 3-5 volumes of 0.1 M glycine (pH 2.5); fractions of approximately 900 µl were collected and neutralized with 100 µl 1 M Tris buffer, pH 8.0. Aliquots of 15 µl were removed from each fraction and boiled in an equal volume of 2X Laemmli sample buffer with 1/15 volume 1 M dithiothreitol (DTT). These samples were electrophoresed on 8% Novex (San Diego, CA) polyacrylamide gels and visualized either by Coomassie stain or by silver stain using a Daiichi kit (Enprotech, Natick, MA) according to the manufacturer's suggested protocol. Fractions which contained the largest amounts of protein were combined and concentrated by vacuum. The remaining solution was diluted by 50% with reducing Laemmli sample buffer and run on 1.5 mm 7% polyacrylamide gels in Tris-glycine/SDS buffer. Protein was transferred from the gels to Immobilon membrane by the procedure described in Example 2 using the Hoefer transfer apparatus.

The protein bands corresponding to canine α_{TM1} were excised from 10 PVDF membranes and resulted in approximately 47 µg total protein. The bands were destained in 4 ml 50% methanol for 5 minutes, air dried and cut into 1 x 2 mm pieces. The membrane pieces were submerged in 2 ml 95% acetone at 4°C for 30 minutes with occasional vortexing and then air dried.

Prior to proteolytic cleavage of the membrane bound protein, 3 mg of cyanogen bromide (CNBr) (Pierce, Rockford, IL) were dissolved in 1.25 ml 70% formic acid. This solution was then added to a tube containing the PVDF membrane pieces and the tube incubated in the dark at room temperature for 24 hours. The supernatant (S1)

was then removed to another tube and the membrane pieces washed with 0.25 ml 70% formic acid. This supernatant (S2) was removed and added to the previous supernatant (S1). Two milliliters of Milli Q water were added to the combined supernatants (S1 and S2) and the solution lyophilized. The PVDF membrane pieces were dried under nitrogen and extracted again with 1.25 ml 60% acetonitrile, 0.1% tetrafluoroacetic acid (TFA) at 42°C for 17 hours. This supernatant (S3) was removed and the membrane pieces extracted again with 1.0 ml 80% acetonitrile with 0.08% TFA at 42°C for 1 hour. This supernatant (S4) was combined with the previous supernatants (S1, S2 and S3) and vacuum dried.

The dried CNBr fragments were then dissolved in 63 µl 8 M urea, 0.4 M NH_4HCO_3. The fragments were reduced in 5 µl 45 mM dithiothreitol (DTT) and subsequently incubated at 50°C for 15 minutes. The solution was then cooled to room temperature and the fragments alkylated by adding 5 µl 100 mM iodoacetamide (Sigma, St. Louis, MO). Following a 15 minute incubation at room temperature, the sample was diluted with 187 µl Milli Q water to a final urea concentration of 2.0 M. Trypsin (Worthington, Freehold, NJ) was then added at a ratio of 1:25 (w:w) of enzyme to protein and the protein digested for 24 hours at 37°C. Digestion was terminated with addition of 30 µl TFA.

The protein fragments were then separated with high performance liquid chromatography (HPLC) on a Waters 625 LC system (Millipore, Milford, MA) using a 2.1 x 250 mm, 5 micron Vydac C-18 column (Vydac, Hesperia, CA) equilibrated in 0.05% TFA and HPLC water (buffer A). The peptides were eluted with increasing concentration of 80% acetonitrile in 0.04% TFA (buffer B) with a gradient of 38-75% buffer B for 65-95 minutes and 75-98% buffer B for 95-105 minutes. Peptides were fractionated at a flow rate of 0.2 ml/minute and detected at 210 nm.

Following fractionation, the amino acid sequence of the peptides was analyzed by automated Edman degradation performed on an Applied Biosystems Model 437A protein sequencer using the manufacturer's standard cycles and the Model 610A Data Analysis software program, Version 1.2.1. All sequencing reagents were supplied by Applied Biosystems. The amino acid sequences of seven of the eight internal fragments are set out below wherein "X" indicates the identity of the amino acid was not certain.

VFQEXGAGFGQ (SEQ ID NO: 15)

LYDXVAATGLXQPI (SEQ ID NO: 16)

PLEYXDVIPQAE (SEQ ID NO: 17)

FQEGFSXVLX (SEQ ID NO: 18)

TSPTFIXMSQENVD (SEQ ID NO: 19)

LVVGAPLEVVAVXQTGR (SEQ ID NO: 20)

LDXKPXDTA (SEQ ID NO: 21)

Primer Design

One internal amino acid sequence (set out in SEQ ID NO: 22) obtained was then used to design a fully degenerate oligonucleotide primer, designated p4(R) as set out in SEQ ID NO: 23.

FGEQFSE (SEQ ID NO: 22)

5′-RAANCCYTCYTGRAAACTYTC-3′ (SEQ ID NO: 23)

Example 4

PCR Cloning Of A Canine α_{TM1} Fragment

The 5′ portion of the canine α_{TM1} gene was amplified from double-stranded canine splenic cDNA by PCR.

Generation of Double Stranded Canine Spleen cDNA

One gram of frozen material from a juvenile dog spleen was ground in liquid nitrogen on dry ice and homogenized in 20 ml RNA-Stat 60 buffer (Tel-Test B, Inc, Friendswood, TX). Four ml chloroform were added, and the solution extracted by centrifugation at 12,000 g for 15 minutes. RNA was precipitated from the aqueous layer with 10 ml ethanol. Poly A^+ RNA was then selected on Dynal Oligo dT Dynabeads® (Dynal, Oslo, Norway). Five aliquots of 100 µg total RNA were combined and diluted with an equal volume of 2X binding buffer (20 mM Tris-HCl, pH 7.5, 1.0 M LiCl, 1 mM EDTA, 0.1% SDS). RNA was then incubated 5 minutes with the Oligo dT Dynabeads® (1.0 ml or 5 mg beads for all the samples). Beads were washed with buffer containing 10 mM Tris-HCl, pH 7.5, 0.15 M LiCl, 1 mM EDTA and 0.1% SDS, according to the

manufacturer's suggested protocol prior to elution of poly A^+ mRNA with 2 mM EDTA, pH 7.5. Double-stranded cDNA was then generated using the eluted poly A^+ mRNA and the Boehringer Mannheim cDNA Synthesis Kit according to the manufacturer's suggested protocol.

Isolation of a Partial Canine α_{TM1} cDNA

Oligonucleotide primers 5'Deg (SEQ ID NO: 9) and p4(R) (SEQ ID NO: 23) were employed in a standard PCR reaction using 150 ng double-stranded cDNA, 500 ng of each primer, 200 μM dNTPs and 1.5 units *Taq* polymerase (Boehringer Mannheim) in *Taq* buffer (Boehringer Mannheim) with magnesium. The resulting products (1 μl of the original reaction) were subjected to a second round of PCR with the same primers to increase product yield. This band was eluted from a 1% agarose gel onto Schleicher & Schuell (Keene, NH) NA45 paper in a buffer containing 10 mM Tris-HCl, pH 8, 1 mM EDTA, 1.5 M NaCl at 65°C, precipitated, and ligated into the pCR™II vector (Invitrogen, San Diego, CA) using the TA cloning kit (Invitrogen) and the manufacturer's suggested protocol. The ligation mixture was transformed by electroporation into XL-1 Blue bacteria (Stratagene). One clone, 2.7, was determined to contain sequences corresponding to α_{TM1} peptide sequences which were not utilized in design of the primers.

Sequencing was performed with an Applied Biosystems 373A DNA sequencer (Foster City, CA) with a Dye-deoxy terminator cycle sequence kit (ABI) in which fluorescent-labeled dNTPs were incorporated in an asymmetric PCR reaction [McCabe, "Production of Single Stranded DNA by Asymmetric PCR," in PCR Protocols: A Guide to Methods and Applications, Innis, *et al.* (eds.) pp. 76-83 Academic Press: New York (1990)] as follows. Samples were held at 96°C for 4 minutes and subjected to 25 cycles of the step sequence: 96°C, for 15 seconds; 50°C for 1 second; 60°C for 4 minutes. Sequence data was automatically down-loaded into sample files on the computer that included chromatogram and text files. The sequence of the entire insert of clone 2.7 is set out in SEQ ID NO: 24.

Attempts to isolate the full length canine α_{TM1} cDNA from the Stratagene library (as described in Example 2) were unsuccessful. Approximately 1 x 10^6 phage

plaques were screened by hybridization under low stringency conditions using 30% formamide with clone 2.7 as a probe, but no positive clones resulted. Attempts to amplify relevant sequences downstream from those represented in clone 2.7 using specific oligonucleotides derived from clone 2.7 or degenerate primers based on amino acid sequence from other peptide fragments paired with a degenerate oligonucleotide based on the conserved α subunit amino acid motif GFFKR [Tamura, *et al., supra*] were also unsuccessful.

Example 5

Cloning Of A Putative Human Homolog Of Canine α_{TM1}

To attempt the isolation of a human sequence homologous to canine α_{TM1} the approximately 1 kb canine α_{TM1} fragment from clone 2.7 was used as a probe. The probe was generated by PCR under conditions described in Example 2 using NT2 (as set out in SEQ ID NO: 25) and p4(R) (SEQ ID NO: 23) primers.

5′-GTNTTYCARGARGAYGG-3′ (SEQ ID NO: 25)

The PCR product was purified using the Qiagen (Chatsworth, GA) Quick Spin kit and the manufacturer's suggested protocol. The purified DNA (200 ng) was labeled with 200 μCi α^{32}PdCTP using the Boehringer Mannheim Random Prime Labelling kit and the manufacturer's suggested protocol. Unincorporated isotope was removed with Sephadex® G25 (fine) gravity chromatography. The probe was denatured with 0.2 N NaOH and neutralized with 0.4 M Tris-HCl, pH 8.0, before use.

Colony lifts on Hybond® filters (Amersham) of a human spleen cDNA library in pCDNA/Amp (Invitrogen, San Diego, CA) were prepared. The filters were initially denatured and neutralized as described in Example 2 and subsequently incubated in a prehybridization solution (8 ml/filter) with 30% formamide at 50°C with gentle agitation for 2 hours. Labeled probe as described above was added to this solution and incubated with the filters for 14 hours at 42°C. The filters were washed twice in 2X SSC/0.1% SDS at 37°C and twice in 2X SSC/0.1% SDS at 50°C. Final stringency washes were 1X SSC/0.1% SDS, twice at 65°C (1X SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0). Filters were exposed to Kodak X-Omat AR film for six hours with an

intensifying screen. Colonies giving signals on duplicate lifts were streaked on LB medium with magnesium (LBM)/carbenicillin plates and incubated overnight at 37°C. Resulting streaked colonies were lifted with Hybond® filters and these filters were treated as above. The filters were hybridized under more stringent conditions with the 1 kb probe from clone 2.7, labeled as previously described, in a 50% formamide hybridization solution at 50°C for 3 hours. Probed filters were washed with a final stringency of 0.1 X SSC/0.1% SDS at 65°C and exposed to Kodak X-Omat AR film for 2.5 hours at -80°C with an intensifying screen. Positive colonies were identified and cultured in LBM/carbenicillin medium overnight. DNA from the cultures was prepared using the Promega Wizard® miniprep kit according to the manufacturer's suggested protocol and the resulting DNA was sequenced.

The initial screening resulted in 18 positive clones, while the secondary screening under more stringent hybridization conditions produced one positive clone which was designated 19A2. The DNA and deduced amino acid sequences of the human α_d clone 19A2 are set out in SEQ ID NOS: 1 and 2, respectively.

Characteristics Of The Human α_d cDNA and Predicted Polypeptide

Clone 19A2 encompasses the entire coding region for the mature protein, plus 48 bases (16 amino acid residues) of the 5′ upstream signal sequence and 241 bases of 3′ untranslated sequence which do not terminate in a polyadenylation sequence. The core molecular weight of the mature protein is predicted to be around 125 kD. The extracellular domain is predicted to encompass approximately amino acid residues 17 through 1108 of SEQ ID NO: 2. This extracellular region is contiguous with about a 20 amino acid region homologous to the human CD11c transmembrane region (residues 1109 through 1128 of SEQ ID NO: 2). The cytoplasmic domain comprises approximately 30 amino acids (about residues 1129 through 1161 of SEQ ID NO: 2). The protein also contains a region (around residues 150 through 352) of approximately 202 amino acids homologous to the I (insertion) domain common to CD11a, CD11b and CD11c [Larson and Springer, *supra*], α_E [Shaw, *et al., J.Biol.Chem. 269*:6016-6025 (1994)] and in VLA-1 and VLA-2, [Tamura, *et al., supra*]. The I domain in other integrins has been shown to participate in ICAM binding [Landis, *et al., J.Cell.Biol. 120*:1519-1527 (1993);

Diamond, *et al., J.Cell.Biol. 120*:1031-1043 (1993)], suggesting that α_d may also bind members of the ICAM family of surface molecules. This region has not been demonstrated to exist in any other integrin subunits.

The deduced amino acid sequence of α_d shows approximately 36% identity to that of CD11a, approximately 60% identity to CD11b and approximately 66% identity to CD11c. An alignment of amino acid sequences for (CD11b SEQ ID NO: 3), CD11c (SEQ ID NO: 4) and α_d (SEQ ID NO: 2) is presented in Figure 1.

The cytoplasmic domains of α subunits in β_2 integrins are typically distinct from one another within the same species, while individual α subunits show high degrees of homology across species boundaries. Consistent with these observations, the cytoplasmic region of α_d differs markedly from CD11a, CD11b, and CD11c except for a membrane proximal GFFKR amino acid sequence which has been shown to be conserved among all α integrins [Rojiani, *et al., Biochemistry 30*: 9859-9866 (1991)]. Since the cytoplasmic tail region of integrins has been implicated in "inside out" signaling and in avidity regulation [Landis *et al., supra*], it is possible that α_d interacts with cytosolic molecules distinct from those interacting with CD11a, CD11b, and CD11c, and, as a result, participates in signaling pathways distinct from those involving other β_2 integrins.

The extracellular domain of α_d contains a conserved DGSGS amino acid sequence adjacent the I-domain; in CD11b, the DGSGS sequence is a metal-binding region required for ligand interaction [Michishita, *et al. Cell 72*:857-867 (1993)]. Three additional putative cation binding sites in CD11b and CD11c are conserved in the α_d sequence at amino acids 465-474, 518-527, and 592-600 in clone 19A2 (SEQ ID NO: 1). The α_d I-domain is 36%, 62%, and 57% identical to the corresponding regions in CD11a, CD11b, and CD11c, respectively, and the relatively low sequence homology in this region suggests that α_d may interact with a set of extracellular proteins distinct from proteins with which other known β_2 integrins interact. Alternatively, the affinity of α_d for known β_2 integrin ligands, for example, ICAM-1, ICAM-2 and/or ICAM-R, may be distinct from that demonstrated for the other β_2 integrin/ICAM interactions. [See Example 12.]

Isolation of additional human α_d cDNA clones for sequence verification

In order to confirm the DNA sequence encoding human α_d, additional human cDNAs were isolated by hybridization from a human splenic oligo dt-primed cDNA library (Invitrogen) in pcDNA/Amp (described in Example 5) which was size selected by agarose gel electrophoresis for cDNA greater than 3 kb in length. The probe for hybridization was derived from a 5′ region of α_d as described below. Hybridization conditions were the same as described above for the isolation of the initial human α_d clone, except that following hybridization, filters were washed twice in 2X SSC/0.1% SDS at room temperature and once in 2X SSC/0.1% SDS at 42°C. Filters were exposed to Kodak X-Omat AR film overnight.

The 5′ α_d hybridization probe was generated by PCR from the 19A2 clone using primers CD11c 5′ For (SEQ ID NO: 94) and CD11c 5′ Rev (SEQ ID NO: 95) under the following conditions. Samples were held at 94°C for four minutes and subjected to 30 cycles of the temperature step sequence i) 94°C, for 15 seconds; ii) 5°C, for 30 seconds; and iii) 72°C, for 1 minute in a Perkin-Elmer 9600 thermocycler.

CD11c 5′ For: (5′)CTGGTCTGGAGGTGCCTTCCTG(3′) (SEQ ID NO: 94)
CD11c 5′ Rev: (5′)CCTGAGCAGGAGCACCTGGCC(3′) (SEQ ID NO: 95)

The amplification product was purified using the BioRad (Hercules, CA) Prep-A-Gene kit according to manufacturer's suggested protocol. The resulting 5′ α_d probe was approximately 720 bases long, corresponding to the region from nucleotide 1121 to nucleotide 1839 in SEQ ID NO: 1. The purified DNA (approximately 50 ng) was labeled with ^{32}P-dCTP using a Boehringer Mannheim (Indianapolis, Indiana) Random Prime Labeling kit according to manufacturer's suggested protocol. Unincorporated isotope was removed using Centrisep® Spin Columns (Princeton Separations, Adelphia, NJ) according to manufacturer's suggested protocol. Labeled probe was added to the filters in a prehybridization solution containing 45% formamide and incubation allowed to proceed overnight at 50°C. Following incubation, the filters were washed as described above.

Thirteen colonies gave signals on duplicate lifts. Positive colonies were

picked from master plates, diluted in LBM and carbenicillin (100 μg/ml) and plated at varying dilutions onto Hybond® (Amersham) filters. Duplicate filters were hybridized with the same solution from the primary hybridization and following hybridization, the filters were washed at a final stringency of 2X SSC/0.1% SDS at 42°C and exposed to film.

Ten of the originally identified thirteen positive colonies were confirmed in the secondary screen. Of these ten clones, two (designated A7.Q and A8.Q) were sequenced and determined to encode human α_d. Clone A7.Q was found to be approximately 2.5 kb in length, including a 5′ leader, part of a coding region, and an additional 60 bases of 5′ untranslated sequence. The incomplete coding region was determined to have resulted from an aberrantly spliced intron region at corresponding nucleotide 2152 of SEQ ID NO: 1. Clone A8.Q was determined to be approximately 4 kb in length, spanning the entire α_d coding region and also including an intron sequence at corresponding base 305 of SEQ ID NO: 1. In comparison to the originally isolated α_d clone (SEQ ID NO: 1), one difference was observed in that both A7.Q and A8.Q clones were determined to have a three base CAG codon insertion occurring at base 1495. Sequences for clones A7.Q AND A8.Q are set out in SEQ ID NOs: 96 and 97, respectively, and a composite human sequence derived from clones A7.Q and A8.Q, and its corresponding deduced amino acid sequence, are set out in SEQ ID NOs: 98 and 99, respectively.

Example 6
Northern Analysis of Human α_d Expression in Tissues

In order to determine the relative level of expression and tissue specificity of α_d, Northern analysis was performed using fragments from clone 19A2 as probes. Approximately 10 μg of total RNA from each of several human tissues or cultured cell lines were loaded on a formaldehyde agarose gel in the presence of 1 μg of ethidium bromide. After electrophoresis at 100 V for 4 hr, the RNA was transferred to a nitrocellulose membrane (Schleicher & Schuell) by wicking in 10X SSC overnight. The membrane was baked 1.5 hr at 80°C under vacuum. Prehybridization solution containing 50% formamide in 3-(N-morpholino)propane sulfonic acid (MOPS) buffer was used to block the membrane for 3 hr at 42°C. Fragments of clone 19A2 were labeled with the

Boehringer Mannheim Random Prime kit according to the manufacturer's instructions including both αP^{32}dCTP and αP^{32}dTTP. Unincorporated label was removed on a Sephadex® G25 column in TE buffer. The membrane was probed with 1.5×10^6 counts per ml of prehybridization buffer. The blot was then washed successively with 2X SSC/0.1% SDS at room temperature, 2X SSC/0.1% SDS at 42°C, 2X SSC/0.1% SDS at 50°C, 1X SSC/0.1% SDS at 50°C, 0.5X SSC/0.1% SDS at 50°C and 0.1X SSC/0.1% SDS at 50°C. The blot was then exposed to film for 19 hr.

Hybridization using a *Bst*XI fragment from clone 19A2 (corresponding to nucleotides 2011 to 3388 in SEQ ID NO: 1) revealed a weak signal in the approximately 5 kb range in liver, placenta, thymus, and tonsil total RNA. No signal was detected in kidney, brain or heart samples. The amount of RNA present in the kidney lane was minimal, as determined with ethidium bromide staining.

When using a second fragment of clone 19A2 (encompassing the region from bases 500 to 2100 in SEQ ID NO: 1), RNA transcripts of two different sizes were detected in a human multi-tissue Northern (MTN) blot using polyA$^+$ RNA (Clontech). An approximately 6.5 kb band was observed in spleen and skeletal muscle, while a 4.5 kb band was detected in lung and peripheral blood leukocytes. The variation in sizes observed could be caused by tissue specific polyadenylation, cross reactivity of the probe with other integrin family members, or hybridization with alternatively spliced mRNAs.

Northern analysis using a third fragment from clone 19A2, spanning nucleotides 2000 to 3100 in SEQ ID NO: 1, gave results consistent with those using the other clone 19A2 fragments.

RNA from three myeloid lineage cell lines was also probed using the fragments corresponding to nucleotides 500 to 2100 and 2000 to 3100 in SEQ ID NO:1. A THP-1 cell line, previously stimulated with PMA, gave a diffuse signal in the same size range (approximately 5.0 kb), with a slightly stronger intensity than the tissue signals. RNA from unstimulated and DMSO-stimulated HL-60 cells hybridized with the α_d probe at the same intensity as the tissue samples, however, PMA treatment seemed to increase the signal intensity. Since PMA and DMSO drive HL-60 cell differentiation toward monocyte/macrophage and granulocyte pathways, respectively, this result suggests enhanced α_d expression in monocyte/macrophage cell types. U937 cells expressed the α_d

message and this signal did not increase with PMA stimulation. No band was detected in Molt, Daudi, H9, JY, or Jurkat cells.

Example 7

Transient Expression of Human α_d Constructs

The human clone 19A2 lacks an initiating methionine codon and possibly some of the 5′ signal sequence. Therefore, in order to generate a human expression plasmid containing 19A2 sequences, two different strategies were used. In the first, two plasmids were constructed in which signal peptide sequences derived from genes encoding either CD11b or CD11c were spliced into clone 19A2 to generate a chimeric α_d sequence. In the second approach, a third plasmid was constructed in which an adenosine base was added at position 0 in clone 19A2 to encode an initiating methionine.

The three plasmids contained different regions which encoded the 5′ portion of the α_d sequence or the chimeric α_d sequence. The α_d region was PCR amplified (see conditions in Example 2) with a specific 3′ primer BamRev (set out below in SEQ ID NO: 26) and one of three 5′ primers. The three 5′ primers contained in sequence: (1) identical nonspecific bases at positions 1-6 allowing for digestion, an *Eco*RI site from positions 7-12 and a consensus Kozak sequence from positions 13-18; (2) a portion of the CD11b (primer ER1B) or CD11c (primer ER1C) signal sequence, or an adenosine (primer ER1D); and (3) an additional 15-17 bases specifically overlapping 5′ sequences from clone 19A2 to allow primer annealing. Primers ER1B, ER1C or ER1D are set out in SEQ ID NOS: 27, 28 or 29, respectively, where the initiating methionine codon is underlined and the *Eco*RI site is double underlined.

5′-CCACTGTCAGGATGCCCGTG-3′ (SEQ ID NO: 26)

5′-AGTTACGAATTCGCCACCATGGCTCTACGGGTGCTTCTTCTG-3′ (SEQ ID NO: 27)

5′-AGTTACGAATTCGCCACCATGACTCGGACTGTGCTTCTTCTG-3′ (SEQ ID NO: 28)

5′-AGTTACGAATTCGCCACCATGACCTTCGGCACTGTG-3′ (SEQ ID NO: 29)

The resulting PCR product was digested with *Eco*RI and *Bam*HI.

All three plasmids contained a common second α_d region (to be inserted immediately downstream from the 5′ region described in the previous paragraph) including the 3′ end of the α_d clone. The second α_d region, which extended from nucleotide 625 into the *Xba*I site in the vector 3′ polylinker region of clone 19A2, was isolated by digestion of clone 19A2 with *Bam*HI and *Xba*I.

Three ligation reactions were prepared in which the 3′ α_d *Bam*HI/*Xba*I fragment was ligated to one of the three 5′ α_d *Eco*RI/*Bam*HI fragments using Boehringer Mannheim ligase buffer and T4 ligase (1 unit per reaction). After a 4 hour incubation at 14°C, an appropriate amount of vector pcDNA.3 (Invitrogen) digested with *Eco*RI and *Xba*I was added to each reaction with an additional unit of ligase. Reactions were allowed to continue for another 14 hours. One tenth of the reaction mixture was then transformed into competent XL-1 Blue cells. The resulting colonies were cultured and the DNA isolated as in Example 5. Digestion with *Eco*RI identified three clones which were positive for that restriction site, and thus, the engineered signal sequences. The clones were designated pATM.B1 (CD11b/α_d, from primer ER1B), pATM.C10 (CD11c/α_d, from primer ER1C) and pATM.D12 (adenosine/α_d from primer ER1d). The presence of the appropriate signal sequences in each clone was verified by nucleic acid sequencing.

Expression from the α_d plasmids discussed above was effected by cotransfection of COS cells with the individual plasmids and a CD18 expression plasmid, pRC.CD18. As a positive control, COS cells were also co-transfected with the plasmid pRC.CD18 and a CD11a expression plasmid, pDC.CD11A.

Cells were passaged in culture medium (DMEM/10%FBS/pen-strep) into 10 cm Corning tissue culture-treated petri dishes at 50% confluency 16 hours prior to transfection. Cells were removed from the plates with Versene buffer (0.5 mM NaEDTA in PBS) without trypsin for all procedures. Before transfection, the plates were washed once with serum-free DMEM. Fifteen micrograms of each plasmid were added to 5 ml transfection buffer (DMEM with 20 μg/ml DEAE-Dextran and 0.5 mM chloroquine) on each plate. After 1.5 hours incubation at 37°C, the cells were shocked for 1 minute with 5 ml DMEM/10% DMSO. This DMSO solution was then replaced with 10 ml/plate culture medium.

Resulting transfectants were analyzed by ELISA, FACS, and immunoprecipitation as described in Examples 8, 9, and 10.

Example 8

ELISA Analysis of COS Transfectants

In order to determine if the COS cells co-transfected with CD18 expression plasmid pRC.CD18 and an α_d plasmid expressed α_d on the cell surface in association with CD18, ELISAs were performed using primary antibodies raised against CD18 (*e.g.*, TS1/18 purified from ATCC HB203). As a positive control, ELISAs were also performed on cells co-transfected with the CD18 expression plasmid and a CD11a expression plasmid, pDC.CD11A. The primary antibodies in this control included CD18 antibodies and anti-CD11a antibodies (*e.g.*, TS1/22 purified from ATCC HB202).

For ELISA, cells from each plate were removed with Versene buffer and transferred to a single 96-well flat-bottomed Corning tissue culture plate. Cells were allowed to incubate in culture media 2 days prior to assay. The plates were then washed twice with 150 µl/well D-PBS/0.5% teleost skin gelatin (Sigma) solution. This buffer was used in all steps except during the development. All washes and incubations were performed at room temperature. The wells were blocked with gelatin solution for 1 hour. Primary antibodies were diluted to 10 µg/ml in gelatin solution and 50 µl were then added to each well. Triplicate wells were set up for each primary antibody. After 1 hour incubation, plates were washed 3X with 150 µl/well gelatin solution. Secondary antibody (goat anti-mouse Ig/HRP-Fc specific [Jackson, West Grove, PA]) at a 1:3500 dilution was added at 50 µl/well and plates were incubated for 1 hour. After three washes, plates were developed for 20 minutes with 100 µl/well *o*-phenyldiamine (OPD) (Sigma) solution (1 mg/ml OPD in citrate buffer) before addition of 50 µl/well 15% sulfuric acid.

Analysis of transfectants in the ELISA format with anti-CD18 specific antibodies revealed no significant expression above background in cells transfected only with the plasmid encoding CD18. Cells co-transfected with plasmid containing CD11a and CD18 showed an increase in expression over background when analyzed with CD18 specific antibodies or with reagents specific for CD11a. Further analysis of cells co-transfected with plasmids encoding CD18 and one of the α_d expression constructs (pATM.C10 or pATM.D12) revealed that cell surface expression of CD18 was rescued

by concomitant expression of α_d. The increase in detectable CD18 expression in COS cells transfected with pATM.C10 or pATM.D12 was comparable to that observed in co-transfected CD11a/CD18 positive control cells.

Example 9
FACS Analysis of COS Transfectants

For FACS analysis, cells in petri dishes were fed with fresh culture medium the day after transfection and allowed to incubate 2 days prior to the assay. Transfectant cells were removed from the plates with 3 ml Versene, washed once with 5 ml FACS buffer (DMEM/2% FBS/0.2% sodium azide) and diluted to 500,000 cells/sample in 0.1 ml FACS buffer. Ten microliters of either 1 mg/ml FITC-conjugated CD18, CD11a, or CD11b specific antibodies (Becton Dickinson) or 800 µg/ml CFSE-conjugated murine 23F2G (anti-CD18) (ATCC HB11081) were added to each sample. Samples were then incubated on ice for 45 minutes, washed 3X with 5 ml/wash FACS buffer and resuspended in 0.2 ml FACS buffer. Samples were processed on a Becton Dickinson FACscan and the data analyzed using Lysys II software (Becton Dickinson).

COS cells transfected with CD18 sequences only did not stain for CD18, CD11a or CD11b. When co-transfected with CD11a/CD18, about 15% of the cells stained with antibodies to CD11a or CD18. All cells transfected with CD18 and any α_d construct resulted in no detectable staining for CD11a and CD11b. The pATM.B1, pATM.C10 and pATM.D12 groups stained 4%, 13% and 8% positive for CD18, respectively. Fluorescence of the positive population in the CD11a/CD18 group was 4-fold higher than background. In comparison, the co-transfection of α_d constructs with the CD18 construct produced a positive population that showed a 4- to 7-fold increase in fluorescence intensity over background.

Example 10
Biotin-Labeled Immunoprecipitation of
Human α_d/CD18 Complexes from Co-transfected COS Cells

Immunoprecipitation was attempted on cells co-transfected with CD18 and each of the α_d expression plasmids separately described in Example 7 in order to determine if α_d could be isolated as part of the $\alpha\beta$ heterodimer complex characteristic of

integrins.

Transfected cells (1-3 x 10^8 cells/group) were removed from petri dishes with Versene buffer and washed 3 times in 50 ml/group D-PBS. Each sample was labeled with 2 mg Sulpho-NHS Biotin (Pierce, Rockford, IL) for 15 minutes at room temperature. The reaction was quenched by washing 3 times in 50 ml/sample cold D-PBS. Washed cells were resuspended in 1 ml lysis buffer (1% NP40, 50 mM Tris-HCl, pH 8.0, 0.2 M NaCl, 2 mM Ca^{++}, 2 mM Mg^{++}, and protease inhibitors) and incubated 15 minutes on ice. Insoluble material was pelleted by centrifugation at 10,000 g for 5 minutes, and the supernatant removed to fresh tubes. In order to remove material non-specifically reactive with mouse immunoglobulin, a pre-clearance step was initially performed. Twenty-five micrograms of mouse immunoglobulin (Cappel, West Chester, PA) was incubated with supernatants at 4°C. After 2.5 hr, 100 µl (25 µg) rabbit anti-mouse Ig conjugated Sepharose® (prepared from Protein A Sepharose® 4B and rabbit anti-mouse IgG, both from Zymed, San Francisco, CA) was added to each sample; incubation was continued at 4°C with rocking for 16 hours. Sepharose® beads were removed from the supernatants by centrifugation. After pre-clearance, the supernatants were then treated with 20 µg anti-CD18 antibody (TS1.18) for 2 hours at 4°C. Antibody/antigen complexes were isolated from supernatants by incubation with 100 µl/sample rabbit anti-mouse/Protein A-Sepharose® preparation described above. Beads were washed 4 times with 10 mM HEPES, 0.2 M NaCl, and 1% Triton-X 100®. Washed beads were pelleted and boiled for 10 minutes in 20 µl 2X Laemmli sample buffer with 2% β-mercaptoethanol. Samples were centrifuged and run on an 8% prepoured Novex polyacrylamide gel (Novex) at 100 V for 30 minutes. Protein was transferred to nitrocellulose membranes (Schleicher & Schuell) in TBS-T buffer at 200 mAmps for 1 hour. Membranes were blocked for 2 hr with 3% BSA in TBS-T. Membranes were treated with 1:6000 dilution of Strep-avidin horse radish peroxidase (POD) (Boehringer Mannheim) for 1 hour, followed by 3 washes in TBS-T. The Amersham Enhanced Chemiluminescence kit was then used according to the manufacturer's instructions to develop the blot. The membrane was exposed to Hyperfilm® MP (Amersham) for 0.5 to 2 minutes.

Immunoprecipitation of CD18 complexes from cells transfected with pRC.CD18 and either pATM.B1, pATM.C10 or pATM.D12 revealed surface expression

of a heterodimeric species consisting of approximately 100 kD β chain, consistent with the predicted size of CD18, and an α chain of approximately 150 kD, corresponding to α_d.

Example 11
Stable Transfection of Human α_d in Chinese Hamster Ovary Cells

To determine whether α_d is expressed on the cell surface as a heterodimer in association with CD18, cDNAs encoding each chain were both transiently and stably transfected into a cell line lacking both α_d and CD18.

For these experiments, α_d cDNA was augmented with additional leader sequences and a Kozak consensus sequence, as described in Example 7, and subcloned into expression vector pcDNA3. The final construct, designated pATM.D12, was co-transfected with a modified commercial vector, pDC1.CD18 encoding human CD18 into dihydrofolate reductase (DHFR)$^-$ Chinese hamster ovary (CHO) cells. The plasmid pDC1.CD18 encodes a DHFR$^+$ marker and transfectants can be selected using an appropriate nucleoside-deficient medium. The modifications which resulted in pDC1.CD18 are as follows.

The plasmid pRC/CMV (Invitrogen) is a mammalian expression vector with a cytomegalovirus promoter and ampicillin resistance marker gene. A DHFR gene from the plasmid pSC1190-DHFR was inserted into pRC/CMV 5′ of the SV40 origin of replication. In addition, a polylinker from the 5′ region of the plasmid pHF2G-DHF was ligated into the pRC/CMV/DHFR construct, 3′ to the DHFR gene. CD18 encoding sequences are subsequently cloned into the resulting plasmid between the 5′ flanking polylinker region and the bovine growth hormone poly A encoding region.

Surface expression of CD18 was analyzed by flow cytometry using the monoclonal antibody TS1/18. Heterodimer formation detected between α_d and CD18 in this cell line was consistent with the immunoprecipitation described in Example 10 with transient expression in COS cells.

Example 12

Human α_d Binds ICAM-R in a CD18-dependent Fashion

In view of reports that demonstrate interactions between the leukocyte integrins and intercellular adhesion molecules (ICAMs) which mediate cell-cell contact [Hynes, *Cell 69*:11-25 (1992)], the ability of CHO cells expressing α_d/CD18 to bind ICAM-1, ICAM-R, or VCAM-1 was assessed by two methods.

In replicate assays, soluble ICAM-1, ICAM-R, or VCAM-1 IgG1 fusion proteins were immobilized on plastic and the ability of α_d/CD18 CHO transfected cells to bind the immobilized ligand was determined. Transfected cells were labeled internally with calcein, washed in binding buffer (RPMI with 1% BSA), and incubated in either buffer only (with or without 10 ng/ml PMA) or buffer with anti-CD18 monoclonal antibodies at 10 µg/ml. Transfected cells were added to 96-well Immulon® 4 microtiter plates previously coated with soluble ICAM-1/IgG1, ICAM-R/IgG1 or VCAM-1/IgG1 fusion protein, or bovine serum albumin (BSA) as a negative control. Design of the soluble forms of these adhesion molecules is described and fully disclosed in co-pending and co-owned U.S. Patent Application Serial No. 08/102,852, filed August 5, 1993. Wells were blocked with 1% BSA in PBS prior to addition of labeled cells. After washing the plates by immersion in PBS with 0.1% BSA for 20 minutes, total fluorescence remaining in each well was measured using a Cytofluor® 2300 (Millipore, Milford, MA).

In experiments with immobilized ICAMs, α_d/CD18 co-transfectants consistently showed a 3-5 fold increase in binding to ICAM-R/IgG1 wells over BSA coated wells. The specificity and CD18-dependence of this binding was demonstrated by the inhibitory effects of anti-CD18 antibody TS1/18. The binding of cells transfected with CD11a/CD18 to ICAM-1/IgG1 wells was comparable to the binding observed with BSA coated wells. CD11a/CD18 transfected cells showed a 2-3 fold increase in binding to ICAM-1/IgG1 wells only following pretreatment with PMA. PMA treatment of α_d/CD18 transfectants did not affect binding to ICAM-1/IgG1 or ICAM-R/IgG1 wells. No detectable binding of α_d/CD18 transfectants to VCAM-1/IgG1 wells was observed.

Binding of α_d/CD18-transfected cells to soluble ICAM-1/IgG1, ICAM-R/IgG1, or VCAM-1/IgG1 fusion proteins was determined by flow cytometry. Approximately one million α_d/CD18-transfected CHO cells (grown in spinner flasks for

higher expression) per measurement were suspended in 100 µl binding buffer (RPMI and 1% BSA) with or without 10 µg/ml anti-CD18 antibody. After a 20 minute incubation at room temperature, the cells were washed in binding buffer and soluble ICAM-1/IgG1 or ICAM-R/IgG1 fusion protein was added to a final concentration of 5 µg/ml. Binding was allowed to proceed for 30 minute at 37°C, after which the cells were washed three times and resuspended in 100 µl binding buffer containing FITC-conjugated sheep anti-human IgG1 at a 1:100 dilution. After a 30 minute incubation, samples were washed three times and suspended in 200 µl binding buffer for analysis with a Becton Dickinson FACScan.

Approximately 40-50% of the α_d/CD18 transfectants indicated binding to ICAM-R/IgG1, but no binding to ICAM-1/IgG1 or VCAM-1/IgG1 proteins. Pretreatment of transfected cells with PMA has no effect on α_d/CD18 binding to either ICAM-1/IgG1, ICAM-R/IgG1 or VCAM-1/IgG1, which was consistent with the immobilized adhesion assay. Binding by ICAM-R was reduced to background levels after treatment of α_d/CD18 transfectants with anti-CD18 antibody TS1/18.

The collective data from these two binding assays illustrate that α_d/CD18 binds to ICAM-R and does so preferentially as compared to ICAM-1 and VCAM-1. The α_d/CD18 binding preference for ICAM-R over ICAM-1 is opposite that observed with CD11a/CD18 and CD11b/CD18. Thus modulation of α_d/CD18 binding may be expected to selectively affect normal and pathologic immune function where ICAM-R plays a prominent role. Moreover, results of similar assays, in which antibodies immunospecific for various extracellular domains of ICAM-R were tested for their ability to inhibit binding of ICAM-R to α_d/CD18 transfectants, indicated that α_d/CD18 and CD11a/CD18 interact with different domains of ICAM-R.

The failure of CD11a/CD18 to bind ICAM-1/IgG1 or ICAM-R/IgG1 in solution suggests that the affinity of binding between CD11a/CD18 and ICAM-1 or ICAM-R is too low to permit binding in solution. Detection of α_d/CD18 binding to ICAM-R/IgG1, however, suggests an unusually high binding affinity.

In the assays described above, the VCAM-1/Ig fusion protein comprised the seven extracellular immunoglobulin-like domains. The fusion protein was produced in transfected CHO cells and protein yield determined by sandwich ELISA. The seven

domain VCAM-1 fusion protein from CHO cell supernatant was employed without purification and protein yield was found to be extremely low. Because of the low protein yield in the VCAM-1 preparations, α_d/CD18 binding to VCAM-1 was re-examined using a commercial VCAM-1 preparation (R & D Systems, Minneapolis, MN) in order to determine if the low VCAM-1 concentration resulted in undetectable α_d binding.

As before, CHO cells expressing α_d and CD18 were utilized in adhesion assays employing immobilized recombinant adhesion molecules. Using flow cytometry, it was shown that α_d-transfected CHO cells expressed both α_d and CD18 and none of the other β_2 integrins. The transfected CHO cells were also shown to express neither of the two known VCAM-1 binding partner proteins, $\alpha_4\beta_1$ and $\alpha_4\beta_7$. The parental CHO cell line was shown to express no α_4 or β_2 integrins. Binding experiments were carried out essentially as described above.

Results indicated that α_d-transfected CHO cells bound immobilized VCAM-1 at a rate of approximately 14.2% as compared to binding to immobilized BSA at a rate of 7.5% and to immobilized E-selection at a rate of 2.8%. In addition, binding to immobilized VCAM-1 was essentially blocked (3.0% binding) in the presence of a monoclonal antibody specific for the first domain of VCAM-1. The parental CHO cells did not bind either VCAM-1, E-selection or BSA (all binding rates were less than 2%). Binding of the transfected CHO cells also decreased with serial passage of the cells which was consistent with the observed decrease in α_d surface expression over the same time period.

In order to determine if cells which naturally express α_d/CD18 utilize VCAM-1 as a binding partner, peripheral blood eosinophils were isolated and cultured five to seven days in the presence of 10 ng/ml IL-5 in order to increase α_d expression. Flow cytometry indicated that IL-5 incubation increased α_d expression two- to four-fold, but had no effect on α_4 expression.

Results indicated that the cultured eosinophils bound immobilized VCAM-1 at a rate of approximately 28.8% and that the binding was partially inhibited by both an anti-CD18 monoclonal antibody (binding rate 17.1%) and a monoclonal antibody against α_4 (binding rate 18.1%). Contrary to the preliminary results above with low levels and/or impure VCAM-1, these data suggest that $\alpha_d\beta_2$ is a ligand for VCAM-1.

The FACS adhesion assay described above was used to test the binding of an ICAM-R mutant E37A/Ig to CHO cells expressing α_d/CD18. E37A/Ig has been shown to obviate binding to an LFA-1/Ig chimera [Sadhu, *et al., Cell Adhesion and Communication 2*:429-440 (1994)]. The mutant protein was expressed in a soluble form from stably transfected CHO cell line and purified over a Prosep®A column as described by Sadhu, *et al., supra*.

E37A/Ig binding with the α_d/CD18 transfectants was not detected in repeated assays. The mean fluorescence intensity (MFI) of the E37A/Ig chimera detected by FITC-conjugated anti-human antibody was identical to the MFI of the detecting antibody alone, indicating there was no detectable signal above background using the E37A/Ig mutant protein in the assay. Similarly, in an ELISA, carried out as described in Example 14, the E37A/Ig mutant did not appear to bind immobilized α_d/CD18.

α_d Binding to iC3b

Complement component C3 can be proteolytically cleaved to form the complex iC3b, which initiates the alternative pathway of complement activation and leads ultimately to cell-mediated destruction of a target. Both CD11b and CD11c have been implicated in iC3b binding and subsequent phagocytosis of iC3b-coated particles. A peptide fragment in the CD11b I domain has recently been identified as the site of iC3b interaction [Ueda, *et al., Proc.Natl.Acad.Sci.(USA) 91*:10680-10684 (1994)]. The region of iC3b binding is highly conserved in CD11b, CD11c, and α_d, suggesting an α_d/iC3b binding interaction.

Binding of α_d to iC3b is performed using transfectants or cell lines naturally expressing α_d (for example, PMA-stimulated HL60 cells) and iC3b-coated sheep red blood cells (sRBC) in a rosette assay [Dana, *et al., J.Clin.Invest. 73*:153-159 (1984)]. The abilities of α_d/CD18 CHO transfectants, VLA4-CHO transfectants (negative control) and PMA-stimulated HL60 cells (positive control) to form rosettes are compared in the presence and absence of an anti-CD18 monoclonal antibody (for example TS1/18.1).

Example 13
Screening by Scintillation Proximity
Assay ID of Modulators of α_d Binding

Specific inhibitors of binding between the α_d ligands of the present invention and their binding partners (α_d ligand/anti-ligand pair) may be determined by a variety of means, such as scintillation proximity assay techniques as generally described in U.S. Patent No. 4,271,139, Hart and Greenwald, *Mol.Immunol. 12*:265-267 (1979), and Hart and Greenwald, *J.Nuc.Med. 20*:1062-1065 (1979), each of which is incorporated herein by reference.

Briefly, one member of the α_d ligand/anti-ligand pair is bound to a solid support either directly or indirectly. Indirect capture would involve a monoclonal antibody, directly bound to the support, which recognizes a specific epitope at the C-terminus of the soluble integrin β chain protein. This epitope would be either the hemagglutinin protein or the mycobacterial IIIE9 epitope [Anderson, *et al., J.Immunol. 141*:607-613 (1988). A fluorescent agent is also bound to the support. Alternatively, the fluorescent agent may be integrated into the solid support as described in U.S. Patent No. 4,568,649, incorporated herein by reference. The non-support bound member of the α_d ligand/anti-ligand pair is labeled with a radioactive compound that emits radiation capable of exciting the fluorescent agent. When the ligand binds the radiolabeled anti-ligand, the label is brought sufficiently close to the support-bound fluorescer to excite the fluorescer and cause emission of light. When not bound, the label is generally too distant from the solid support to excite the fluorescent agent, and light emissions are low. The emitted light is measured and correlated with binding between the ligand and the anti-ligand. Addition of a binding inhibitor to the sample will decrease the fluorescent emission by keeping the radioactive label from being captured in the proximity of the solid support. Therefore, binding inhibitors may be identified by their effect on fluorescent emissions from the samples. Potential anti-ligands to α_d may also be identified by similar means.

The soluble recombinant α_d/CD18 leucine zipper construct (see Example 14) is used in a scintillation proximity assay to screen for modulators of CAM binding by the following method. The recombinant integrin is immobilized with a nonblocking anti-α subunit or anti-β subunit antibody previously coated on a scintillant-embedded plate. Chemical library compounds and a specific biotinylated CAM/Ig chimera are added to the

plate simultaneously. Binding of the CAM/Ig chimera is detected by labeled strepavidin. In the assay, ICAM-1/Ig and ICAM-R/Ig are biotinylated with NHS-Sulfo-biotin LC (long chain, Pierce) according to manufacturer's suggested protocol. Labeled proteins are still reactive with CAM specific antibodies and can be shown to react with immobilized LFA-1 by ELISA, with detection by Strepavidin-HRP and subsequent development with OPD.

Alternatively, the recombinant leucine zipper protein is purified, or partially purified and coated directly on the scintillant embedded plate. Unlabelled CAM/Ig chimera and chemical library compounds are added simultaneously. Bound CAM/Ig is detected with ^{125}I-labeled anti-human Ig.

As yet another alternative, purified CAM/Ig protein is immobilized on the scintillant plate. Chemical library compounds and concentrated supernatant from cells expressing recombinant leucine zipper integrin are added to the plate. Binding of the recombinant integrin is detected with a labeled, non-blocking α or β subunit antibody.

Screening for Small Molecule Modulators

As an alternative to scintillation proximity assays, α_d binding partners and inhibitors of the same can be identified using ELISA-like assays as described below.

Soluble α_d/CD18 leucine zipper (LZ) construct (see Example 14) was captured from tissue culture supernatants using the anti-α_d antibody 212D (see Example 15). The 212D antibody was immobilized on 96-well Immulon® IV plates (Costar) in bicarbonate coating buffer (pH 9.5) overnight at 4°C. The same protocol was used to immobilize the anti-CD11a antibody TS2/4.1 for immobilization of a LFA-1 leucine zipper (LFA-1LZ) fusion protein; LFA-1 was used as a negative control for VCAM-1 binding and a positive control for ICAM-1 binding. The plates were blocked with 300 µl/well 3% bovine serum albumin for one hour and washed in D-PBS. Tissue culture supernatants from stable CHO transfectants expressing either α_d/CD18LZ or LFA-1LZ were added at 100 µl/well and allowed to incubate for 6 to 8 hours at 4°C. The plates were washed twice with Tris-buffered saline with Tween® 20 (TBS-T), followed by one wash with TBS (no Tween®) containing 2 mM each calcium chloride, magnesium chloride, and manganese chloride. The latter served as assay and wash buffer during the remainder of the assay.

After integrin capture, the plates were washed three times with 250 µl/well TBS. Purified CAM/Ig (see Example 12) was added to each well following serial 2:3 dilutions starting at a concentration of 10 to 20 µg/ml. CAM/Igs were allowed to bind for two hours at room temperature before plates were washed as above. Bound fusion protein was detected with horseradish peroxidase-conjugated goat anti-human Ig antibody (Jackson Labs) followed by development with o-phenyldiamine (OPD).

Results indicated that while ICAM-1/Ig caused a 5- to 7-fold increase in signal when bound to LFA-1LZ, it failed to bind α_d/CD18LZ. In contrast, VCAM-1/Ig exhibited a 5-fold increase in signal above background in wells containing α_d/CD18LZ, but not in wells with LFA-1LZ. An ICAM-R mutant E37A/Ig (see Example 12) did not bind either integrin.

The α_d specific monoclonal antibodies 212D, 217L, 217I, 217H, 217G, 217K, and 217M were tested for the ability to inhibit VCAM-1 binding to immobilized α_d/CD18. In addition, anti-VCAM-1 monoclonal antibodies 130K, 130P and IG11B1 (Caltag) were used to determine reaction specificity. The anti-α_d monoclonal antibodies were used at 5 µg/ml and the anti-VCAM-1 antibodies at 25 µg/ml; the higher anti-VCAM-1 antibody concentration was used in view of the fact that VCAM-1 is in solution in the assay system.

Partial blocking (50%) resulted in the wells treated with either 217I or 130K and 130P used together. The combination of 130K/130P also completely inhibits the interaction of VLA-4 and VCAM-1 which suggested that α_d and VLA-4 bind to distinct sites on VCAM-1 and the possibility of developing antagonists which selectively interfere with α_d/VCAM-1 binding.

This assay can be adapted as follows to perform high throughput screening assays for inhibitors of α_d binding. VCAM-1/Ig is biotinylated and used as above in the presence of pooled chemical compounds previously solubilized in DMSO; bound VCAM-1/Ig is then detected with a strepavidin-europium (Eu) complex. The strepavidin-Eu complex is activated by chelation resulting in measurable light emission. Changes, or more particularly a decrease, in emission is indicative of inhibition of VCAM-1/α_d binding, presumably as a result of action by one or more compounds in the pool of small molecules, which are then assayed individually or in smaller groupings.

Example 14
Soluble Human α_d Expression Constructs

The expression of full-length, soluble human α_d/CD18 heterodimeric protein provides easily purified material for immunization and binding assays. The advantage of generating soluble protein is that it can be purified from supernatants rather than from cell lysates (as with full-length membrane-bound α_d/CD18); recovery in therefore improved and impurities reduced.

The soluble α_d expression plasmid was constructed as follows. A nucleotide fragment corresponding to the region from bases 0 to 3161 in SEQ ID NO: 1, cloned into plasmid pATM.D12, was isolated by digestion with *Hind*III and *Aat*II. A PCR fragment corresponding to bases 3130 to 3390 in SEQ ID NO: 1, overlapping the *Hind*III/*Aat*II fragment and containing an addition *Mlu*I restriction site at the 3′ terminus, was amplified from pATM.D12 with primers sHAD.5 and sHAD.3 set out in SEQ ID NOS: 30 and 31, respectively.

5′-TTGCTGACTGCCTGCAGTTC-3′ (SEQ ID NO: 30)

5′-GTTCTGACGCGTAATGGCATTGTAGACCTCGTCTTC-3′ (SEQ ID NO: 31)

The PCR amplification product was digested with *Aat*II and *Mlu*I and ligated to the *Hind*III/*Aat*II fragment. The resulting product was ligated into *Hind*III/*Mlu*I-digested plasmid pDC1.s.

This construct is co-expressed with soluble CD18 in stably transfected CHO cells, and expression is detected by autoradiographic visualization of immunoprecipitated CD18 complexes derived from ^{35}S-methionine labeled cells. The construct is also co-expressed with CD18 in 293 cells [Berman, *et al., J.Cell.Biochem. 52*:183-195 (1993)].

Soluble full-length α_d construct

Alternative α_d expression constructs are also contemplated by the invention. In order to facilitate expression and purification of an intact α_d/CD18 heterodimer, soluble α_d and CD18 expression plasmids will be constructed to include a

"leucine zipper" fusion sequence which should stabilize the heterodimer during purification [Chang, *et al.*, *Proc.Natl.Acad.Sci.(USA)*, *91*: 11408-11412 (1994)]. Briefly, DNA encoding the acidic and basic amino acid strands of the zipper have been generated by primer annealing using oligonucleotides described in Chang, *et al.*. The DNA sequences have been further modified to include additional *Mlu*1 and *Xba*1 restriction sites at the 5′ and 3′ ends, respectively, of the DNA to facilitate subcloning into α_d or CD18 expression constructs previously described. In addition, sequences representing either hemagglutinin protein or a polyhistidine sequence have been added, as well as a stop codon inserted after the *Xba*1 site. The hemagglutinin or polyhistidine sequences are incorporated to facilitate affinity purification of the expressed protein. Sequences encoding the basic strand of the zipper are incorporated on the plasmid vector expressing CD18; the acidic strand is inserted on the α chain construct. Upon expression of the modified α_d and CD18 proteins in a host cell, it is presumed that interaction between the acidic and basic strands of the zipper structure will stabilize the heterodimer and permit isolation of the intact α_d/CD18 molecule by affinity purification as described above.

Plasmids were constructed for expression of soluble α_d and CD18 with acidic and basic "leucine zipper" sequences and transfected into COS cells by the DEAE/Dextran method described in Example 7. The resulting protein was referred to as α_d/CD18LZ. Hemagglutinin and polyhistidine tags were not incorporated into α_d/CD18LZ. Transfected cells were grown for 14 days in reduced serum (2%) conditions. Supernatants harvested every five days from transfected cells were assayed for protein production by ELISA as described in Example 8. Briefly, the α_d/CD18LZ heterodimer was immobilized on plates coated with anti-α_d monoclonal antibody 169B (see Example 15). The α_d/CD18LZ complex was detected by addition of a biotinylated anti-CD18 monoclonal antibody, TS1/18.1 (see Example 8), followed by addition of strepavidin/horse radish peroxidase (HRP) conjugate and o-phenyldiamine (OPD). Protein was clearly detectable in the supernatants.

Binding Assays Using Soluble Full Length α_d Expression Products

Functional binding assays using the soluble full length α_d/CD18LZ heterodimer described above were performed by immobilizing the heterodimer on plates

coated with monoclonal antibody 169B or a non-blocking anti-CD18 monoclonal antibody (see Example 15). Wells were blocked with fish skin gelatin to prevent non-specific binding before addition of CAM/Ig chimeras (see Example 12) at a starting concentration of 10 μg/ml. Binding of the chimeras to α_d/CD18 was detected with a goat-anti-human Ig HRP conjugate (Jackson Labs) and subsequent development with OPD.

VCAM-1/Ig was observed to bind to captured α_d/CD18LZ at a 3-5 fold higher level than to captured CD11a/CD18. ICAM-1/Ig and ICAM-2/Ig bound soluble CD11a/CD18 heterodimer approximately 15 and 10 fold above background, respectively, but did not bind α_d/CD18. VCAM-1 binding was reduced approximately 50% in the presence of the VCAM-1 specific antibodies 130K and 130P used in combination.

The binding assay was also performed with the ICAM/Ig protein immobilized on 96-well plates followed by addition of recombinant soluble integrin in cellular supernatant. Binding of the soluble integrins were detected with an unlabeled non-blocking α or β subunit specific murine antibody, followed by incubation with HRP-conjugated goat anti-mouse antibody and development with OPD.

Results indicated that a non-blocking antibody detected α_d/CD18LZ binding to ICAM-R/Ig 10 fold greater than binding detected in control well containing no antibody. Soluble α_d/CD18 binding was not detected with immobilized ICAM-1/Ig, however binding was detected between α_d/CD18 and immobilized CD11b/CD18 and CD11a/CD18 15 and 5 fold, respectively, greater than background binding.

Because previous studies have demonstrated that CD11b and CD11c bind lipopolysaccharide (LPS) [Wright, *Curr.Opin.Immunol.* 3:83-90 (1991); Ingalls and Golenbock, *J.Exp.Med.* 181:1473-1479 (1995)], LPS binding to α_d/CD18 was also assessed using flow cytometry and plate-based assays. Results indicated that FITC-labelled LPS isolated from *S.Minnesota* and *S.typhosa* (both obtained from Sigma) at 20 μg/ml were able to weakly bind α_d/CD18 transfected CHO cells. No binding was observed with un-transfected control CHO cells. In ELISA format assays, biotinylated LPS [Luk, *et al., Alan. Biochem. 232*:217-224 (1995)] at 0.5 - 3.0 μg bound immobilized α_d/CD18LZ with a signal four fold greater that the capture antibody and blocking reagent alone. Apparent binding of LPS to CD11a/CD18 was discounted by subtracting from each experimental value background binding to anti-CD11a antibody TS2/4.

In order to identify other ligands for α_d/CD18, the recombinant α_d/CD18LZ protein is used in a two tier study. Binding of various cell types to immobilized protein is used to determine which cells express α_d ligands on the cell surface. Antibody inhibition is then used to determine if the observed cell binding results from interaction with known surface adhesion molecules. If no inhibition results, co-immunoprecipitation with α_d/CD18LZ bound to proteins from lysates of cells which will bind α_d is used to attempt to identify the ligand.

Soluble Human α_d I Domain Expression Constructs

It has previously been reported that the I domain in CD11a can be expressed as an independent structural unit that maintains ligand binding capabilities and antibody recognition [Randi and Hogg, *J.Biol.Chem. 269*:12395-12398 (1994); Zhout, *et al., J.Biol.Chem. 269*:17075-17079 (1994); Michishita, *et al., Cell 72*:857-867 (1993)]. To generate a soluble fusion protein comprising the α_d I domain and human IgG4, the α_d I domain is amplified by PCR using primers designed to add flanking *Bam*HI and *Xho*I restriction sites to facilitate subcloning. These primers are set out in SEQ ID NOS: 32 and 33 with restriction sites underlined.

5′-ACGTATGCAGGATCCCATCAAGAGATGGACATCGCT-3′ (SEQ ID NO: 32)

5′-ACTGCATGTCTCGAGGCTGAAGCCTTCTTGGGACATC-3′ (SEQ ID NO: 33)

The C nucleotide immediately 3′ to the *Bam*HI site in SEQ ID NO: 32 corresponds to nucleotide 435 in SEQ ID NO: 1; the G nucleotide 3′ to the *Xho*I site in SEQ ID NO: 33 is complementary to nucleotide 1067 in SEQ ID NO: 1. The amplified I domain is digested with the appropriate enzymes, the purified fragment ligated into the mammalian expression vector pDCs and the prokaryotic expression vector pGEX-4T-3 (Pharmacia) and the I domain fragment sequenced. The fusion protein is then expressed in COS, CHO or *E.coli* cells transfected or transformed with an appropriate expression construct.

Given the affinity of α_d for ICAM-R, expression of the α_d I domain may be of sufficient affinity to be a useful inhibitor of cell adhesion in which α_d participates.

Analysis of Human α_d I Domain/IgG4 Fusion Proteins

Protein was resolved by SDS-PAGE under reducing and non-reducing conditions and visualized by either silver staining or Coomassie staining. Protein was then transferred to Immobilon PVDF membranes and subjected to Western blot analysis using anti-human IgG monoclonal antibodies or anti-bovine Ig monoclonal antibodies.

Protein detected was determined to migrate at about 120 kD under non-reducing conditions and at about 45 kD under reducing conditions. Minor bands were also detected on non-reducing gels at approximately 40-50 kD which were reactive with the anti-human, but not anti-bovine, antibodies. A 200 kD minor band was determined to be bovine Ig by Western blot.

Binding Assays Using I Domain Expression Products

The ability of the I domain to specifically recognize ICAM-R/IgG chimeric protein was tested in an ELISA format. Serial dilutions of α_d I domain IgG4 fusion protein (Iα_d/IgG4) in TBS were incubated with ICAM-1/IgG, ICAM-R/IgG, VCAM-1/IgG, or an irrelevant IgG1 myeloma protein immobilized on Immulon® IV RIA/EIA plates. CD11a I domain/IgG chimeric protein and human IgG4/kappa myeloma protein were used as negative controls. Bound IgG4 was detected with the biotinylated anti-IgG4 monoclonal antibody HP6023 followed by addition of strepavidin-peroxidase conjugate and development with substrate *o*-phenyldiamine.

In repeated assays, no binding of the CD11a/IgG4 protein or the IgG4 myeloma protein was detected with any of the immobilized proteins. The Iα_d/IgG4 protein did not bind to fish skin gelatin or bovine serum albumin blocking agents, human IgG1, or ICAM-1/IgG. A two to three fold increase in binding signal over background was detected in ICAM-R/IgG protein coated wells using 1-5 μg/ml concentrations of Iα_d/IgG4 protein. The signal in VCAM-1/IgG protein coated wells was 7-10 fold higher than background. In previous assays, α_d/CD18 transfected CHO cells did not bind VCAM-1/IgG protein, suggesting that VCAM-1 binding may be characteristic of isolated I domain amino acid sequences.

Additional α_d I domain constructs

Additional α_d I domain constructs are generated in the same fashion as the previous construct, but incorporating more amino acids around the α_d I domain. Specific constructs include: i) sequences from exon 5 (amino acids 127-353 in SEQ ID NO: 2), preceding the current construct, ii) the EF-hand repeats (amino acids 17-603 in SEQ ID NO: 2) following the I domain, and iii) the alpha chain truncated at the transmembrane region (amino acids 17-1029 in SEQ ID NO: 2), with an IgG4 tail for purification and detection purposes. These constructs are ligated into either the mammalian expression vector pDCS1 or the prokaryotic expression vector pGEX-4T-3 (Pharmacia) and the I domain sequenced. The fusion proteins are then be expressed in COS, CHO, or *E.coli* cells transformed or transfected with an appropriate expression construct. Protein are purified on a ProSepA® column (Bioprocessing Limited, Durham, England), tested for reactivity with the anti-IgG4 monoclonal antibody HP6023 and visualized on polyacrylamide gels with Coomassie staining.

In order to construct an expression plasmid for the entire α_d polypeptide, pATM.D12, described *supra*, is modified to express an α_d-IgG4 fusion protein by the following method. IgG4 encoding DNA is isolated from the vector pDCS1 by PCR using primers which individually incorporate a 5′ *Aat*II restriction site (SEQ ID NO: 89) and a 3′ *Xba*I restriction site (SEQ ID NO: 90).

5′-CGCTGTGACGTCAGAGTTGAGTCCAAATATGG-3′ (SEQ ID NO: 89)

5′-GGTGACACTATAGAATAGGGC-3′ (SEQ ID NO: 90)

Plasmid pATM.D12 is digested with *Aat*II and *Xba*I, and the appropriately digested and purified IgG4 PCR product ligated into the linear vector.

Example 15
Production of Human α_d- Specific Monoclonal Antibodies

1. Transiently transfected cells from Example 7 were washed three times in Dulbecco's phosphate buffered saline (D-PBS) and injected at 5 x 10^6 cells/mouse into Balb/c mice with 50 µg/mouse muramyl dipeptidase (Sigma) in PBS. Mice were injected two more times in the same fashion at two week intervals. The pre-bleed and immunized serum from the mice were screened by FACS analysis as outlined in Example 9 and the

spleen from the mouse with the highest reactivity to cells transfected with α_d/CD18 was fused. Hybridoma culture supernatants were then screened separately for lack of reactivity against COS cells transfected with CD11a/CD18 and for reactivity with cells co-transfected with an α_d expression plasmid and CD18.

This method resulted in no monoclonal antibodies.

2. As an alternative for production of monoclonal antibodies, soluble α_d I domain/IgG4 fusion protein was affinity purified from supernatant of stably transfected CHO cells and used to immunize Balb/c mice as described above. Hybridomas were established and supernatants from these hybridomas were screened by ELISA for reactivity against α_d I domain fusion protein. Positive cultures were then analyzed for reactivity with full length α_d/CD18 complexes expressed on CHO transfectants.

Mouse 1908 received three initial immunizations of α_d/CD18 transfected CHO cells and two subsequent boosts with soluble α_d/CD18 heterodimer. Two final immunizations included 50 µg/mouse α_d I domain/IgG4 fusion protein. The fusion produced 270 IgG-producing wells. Supernatant from 45 wells showed at least 7-fold higher binding to Iα_d/IgG4 fusion protein than to human IgG4 by ELISA. None of the supernatants reacted to α_d/CD18 transfected CHO cells as determined by FACS analysis.

To determine whether the supernatants were able to recognize integrin alpha subunit proteins in another context, fresh frozen splenic sections were stained with supernatants from 24 of the 45 wells. Three supernatants were determined to be positive: one stained large cells in the red pulp, while two others stained scattered cells in the red pulp and also trabeculae.

These supernatants were further analyzed by their ability to immunoprecipitate biotinylated CD18 complexes from either α_d/CD18 transfected CHO cells or PMA-stimulated HL60 cells. Fusion wells with supernatants that recognized protein in detergent lysates (which should not be as conformationally constrained as protein expressed as heterodimers) were selected for further subcloning. Monoclonal antibodies which recognize protein in detergent may be more useful in immunoprecipitation of heterodimeric complexes from transfectants, tissues, and cell lines.

3. As another alternative to monoclonal antibody production, CD18 complexes were immunoprecipitated from human spleen lysates with the anti-CD18 monoclonal antibody 23F2G after preclearance of CD11a/CD18 (using monoclonal antibody TS2/4) and CD11b/CD18 (using monoclonal antibody Mo-1). Five Balb/c mice, ten to twelve weeks old, were immunized by subcutaneous injection with approximately 30 μg of resulting protein in complete Freund's adjuvant on day 0, followed by two boosts of 30 ug immunogen/mouse on days 28 and 43 in incomplete Freund's adjuvant. Test sera were drawn ten days following the final boost and reactivity was assessed by using 1:500 dilution of each serum to detect 1 μg/lane immunogen in a Western blot. Sera from three mice detected bands of approximately 95 and 150 kD; no signal was seen in lanes treated with a 1:50 dilution of preimmune sera. The 150 kD band was presumed to represent α_d in an *in vivo* glycosylation state. In addition, all post immune sera immunoprecipitated protein from lysates of biotinylated α_d/CD18 CHO cells that migrated at appropriate molecular weights on SDS-PAGE to represent the heterodimer. From these results, mouse #2212 was selected and was further immunized by intraperitoneal injection on day 64 with 30 μg immunogen in PBS. The mouse was sacrificed four days later, and the spleen was sterilely removed.

A single-cell suspension was formed by grinding the spleen between the frosted ends of two glass microscope slides submerged in serum-free RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin (RPMI) (Gibco, Canada). The cell suspension was filtered through a sterile 70-mesh Nitex cell strainer (Becton Dickinson, Parsippany, New Jersey), and the filtrate washed twice by centrifugation at 200 x *g* for 5 minutes. The resulting pellet was resuspended in 20 ml serum-free RPMI. Thymocytes taken from three naive Balb/c mice were prepared in a similar manner.

Prior to fusion, NS-1 myeloma cells, kept in log phase in RPMI with 10% Fetalclone serum (FCS) (Hyclone Laboratories, Inc., Logan, Utah) for three days prior to fusion, were pelleted by centrifugation at 200 x *g* for 5 minutes, washed twice as described in the foregoing paragraph, and counted. Approximately 2 x 10^8 spleen cells were combined with 4 x 10^7 NS-1 cells, and the resulting mixture pelleted by centrifugation at 200 x *g*. The supernatant was discarded. The cell pellet dislodged by

tapping the tube and 2 ml of 50% PEG 1500 in 75 mM Hepes (pH 8.0, 37°C) (Boehringer Mannheim) was added over the course of one minute with stirring. An additional 14 ml of serum-free RPMI was subsequently added over the next seven minutes, followed by immediate addition of 16 ml RPMI. The resulting mixture was centrifuged at 200 x *g* for 10 minutes and the supernatant was discarded. The pellet was resuspended in 200 ml RPMI containing 15% FBS, 100 mM sodium hypoxanthine, 0.4 mM aminopterin, 16 mM thymidine (HAT) (Gibco), 25 units/ml IL-6 (Boehringer Mannheim) and 1.5 x 10^6 thymocytes/ml, and dispensed into ten 96-well flat bottom tissue culture plates (Corning, United Kingdom) at 200 μl/well. Cells were fed on days 2, 4, and 6 days post-fusion by aspirating approximately 100 μl from each well with an 18 G needle (Becton Dickinson), and adding 100 μl/well plating medium described above, except containing 10 units/ml IL-6 and lacking thymocytes.

On day 7-10 post-fusion, supernatant from each well was screened by antibody capture ELISA, testing for the presence of mouse IgG. Immulon® 4 plates (Dynatech, Cambridge, Massachusetts) were coated with 50 μl/well goat anti-mouse IgA, IgG, or IgM (Organon Teknika) diluted 1:5000 in 50 mM carbonate buffer, pH 9.6, at 4°C. Plates were washed 3X with PBS containing 0.5% Tween® 20 (PBST) and 50 μl culture supernatant from each well was added. After incubation at 37°C for 30 minutes, wells were washed with PBST as above, and 50 μl of horseradish peroxidase conjugated goat anti-mouse IgG(fc) (Jackson ImmunoResearch, West Grove, Pennsylvania) diluted 1:3500 in PBST was added to each well. Plates were incubated as above, washed 4X with PBST and 100 μl substrate, consisting of 1 mg/ml *o*-phenylene diamine (Sigma) and 0.1 μl/ml 30% H_2O_2 in 100 mM Citrate, pH 4.5, was added. The color reaction was stopped after five minutes with addition of 50 μl 15% H_2SO_4. Absorbance at 490nm was determined for each well using a plate reader (Dynatech).

Hybridomas were further characterized as follows. Supernatants from IgG-producing cultures were analyzed by flow cytometry for reactivity to α_d/CD18-transformed CHO cells but not to JY cells (a B-cell line positive for LFA-1, but not other β_2 integrins as observed in previous in-house staining experiments). Briefly, 5 x 10^5 α_d/CD18-transformed CHO or α_d/CD18$^-$ JY cells were suspended in 50 μl RPMI containing 2% FBS and 10 mM NaN_3 (FACS buffer). Individual cell suspensions were

added to 50 μl IgG positive hybridoma culture supernatant in wells of 96-well round bottomed plates (Corning). After a 30 minute incubation on ice, cells were washed twice by pelleting in a clinical centrifuge, supernatant from each well was discarded, and pellets resuspended in 200-300 μl FACS buffer. The last wash was replaced with 50 μl/well of a 1:100 dilution of a $F(ab')_2$ fragment of sheep anti-mouse IgG (H + L)-FITC conjugate (Sigma, St. Louis, Missouri) prepared in FACS Buffer. After incubation as described above, cells were washed twice with Dulbecco's PBS (D-PBS) supplemented with 10 mM NaN_3, and finally resuspended in D-PBS containing 1% paraformaldehyde. Samples were then transferred to polystyrene tubes for flow cytometric analysis (FACS) with a Becton Dickinson FACsan analyzer.

The fusion yielded four cultures deemed positive by both criteria. When the secondary screen was repeated on expanded supernatants approximately four days later, three of the four cultures remained positive. The three wells, designated 169A, 169B, 169D were cloned two to three times, successively, by doubling dilution in RPMI, 15% FBS, 100 mM sodium hypoxanthine, 16 mM thymidine, and 10 units/ml IL-6. Wells of clone plates were scored visually after four days and the number of colonies in the least dense wells were recorded. Selected wells of the each cloning were assayed by FACS after 7-10 days. Activity was found in two of the cultures, 169A and 169B. In the final cloning, positive wells containing single colonies were expanded in RPMI with 11% FBS. Antibody from clonal supernatants of 169A and 169B were isotyped using IsoStrip kit (Boehringer Mannheim) according to manufacturer instructions and found to be of the IgG1 isotype.

Immunoprecipitation of α_d/CD18 complexes from CHO transfectants and PMA-stimulated HL60 cells was used as a tertiary screen for specificity. Hybridomas 169A and 169B precipitated appropriate bands from CHO lines, and a single α chain species of 150-160 kD from HL60 cells as determined by SDS-PAGE. Hybridomas 169A and 169B were deposited May 31, 1995 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 and assigned Accession Numbers HB11907 and HB11906, respectively.

In order to more fully characterize binding properties of 169A and 169B, the ability of each antibody to inhibit binding of the other or the anti-CD18 antibody

TS1/18.1 to soluble α_d/CD18 was tested. Soluble full length α_d/CD18 was immobilized by each unlabeled antibody separately in a 96-well plate format, and biotinylated antibodies were used to detect protein bound by the same or different unlabeled antibodies. Binding was detected using a goat anti-mouse Ig/HRP conjugate followed by addition of OPD substrate. Results indicated that antibody 169A was able to block binding of biotinylated 169A and TS1/18.1, while the antibody 169B blocked binding only of itself.

4. Another mouse (#2214), immunized by the same protocol as mouse #2212, was selected and further immunized by a pre-fusion boost on day 70 with 30 µg purified α_d from spleen lysates in PBS. The mouse was sacrificed four days later, and the spleen was sterilely removed.

The fusion and cloning of positive cells were carried out as described above. The fusion produced five anti-α_d monoclonal hybridomas designated 170D, 170F, 170E, 170X, and 170H which were isotyped as IgG_1 using the IsoStrip kit (Boehringer Mannheim) according to the manufacturer's instructions.

5. Still another mouse, #2211, immunized by the same initial protocol as mouse #2212 and mouse #2214, was selected and further immunized on day 88 with 30 µg immunogen and a pre-fusion boost of 30 µg immunogen on day 203. The mouse was sacrificed four days later, and the spleen was removed and fusion carried out as described above. Hybridoma supernatant was screened by antibody capture ELISA and by flow cytometry as detailed in the above paragraphs.

Fifteen positive hybridomas were identified, designated 188A, 188B, 188C, 188E, 188F, 188G, 188I, 188J, 188K, 188L, 188M, 188N, 188P, 188R and 188T, and isotyped in an ELISA assay. Briefly, Immulon® 4 plates (Dynatech, Cambridge, Massachusetts) were coated at 4°C with 50 µl/well goat anti-mouse IgA,G,M (Organon Teknika) diluted 1:5000 in 50 mM carbonate buffer, pH 9.6. Plates were blocked for 30 minutes at 37°C with 1% BSA in PBS, washed three times with PBS/0.05% Tween® 20 (PBST) and 50 µl culture supernatant (diluted 1:10 in PBST) added. After incubation and washing as above, 50 µl of horseradish peroxidase conjugated rabbit anti-mouse IgG_1, G_{2a},

or G_3 (Zymed, San Francisco, California), diluted 1:1000 in PBST with 1% normal goat serum, was added. Plates were incubated as above, washed four times with PBST, after which 100 µl substrate, consisting of 1 mg/ml o-phenylene diamine (Sigma) and 0.1 µl/ml 30% H_2O_2 in 100 mM citrate, pH 4.5, was added. The color reaction was stopped in 5 minutes with the addition of 50 µl of 15% H_2SO_4. A_{490} was read on a plate reader (Dynatech) and all fifteen antibodies were determined to be IgG1.

The excess spleen cells from mouse #2211 were frozen in a cryovial and stored in liquid nitrogen. The cryovial was thawed quickly by placing into a 37°C water bath, and moving it in a circular motion just until contents were melted. Cells were transferred to a 15 ml centrifuge tube where warm RPMI containing 11% FBS was added slowly 1 ml at a time, allowing three to five minutes between additions. Another 5 ml warm RPMI was added and after a five minute wait, the tube was centrifuged at 200 x *g* for five minutes and supernatant aspirated. Cells were resuspended in RPMI and a fusion carried out as described above. Hybridoma supernatant was screened by antibody capture and flow cytometry as described above.

The fusion yielded five clones designated 195A, 195C, 195D, 195E and 195H. The clones were isotyped by the ELISA procedure as described above; monoclonal antibodies 195A, 195C, 195D and 195E were determined to be IgG_1 and 195H was determined to be IgG_{2a}.

6. In another attempt to generate anti-α_d monoclonal antibodies, mouse #2213 was immunized using the same protocol as mice 2214, 2211, and 2212, but also further immunized on day 414 and 441 with 30 µg of human α_d/CD18 leucine zipper (LZ) bound to Sepharose® beads. The immunogen for mouse #2213 was prepared by immunoprecipitating human α_d/CD18LZ (Example 14) with an anti-CD18 monoclonal antibody and protein A Sepharose®. The precipitated complex was resuspended as a slurry at a 1:1 ratio with PBS prior to injection. The mouse was sacrificed four days after the booster immunization. The spleen was removed and a fusion carried out as previously described above.

Positive hybridomas were identified by ELISA using human α_d/CD18LZ immobilized with the $F(ab)'_2$ fragment of a non-blocking anti-CD18 antibody. Briefly, the

$F(ab)'_2$ fragments were coated at 100 ng/well onto Immulon® 4 ELISA plates overnight at 4°C. After the buffer was aspirated, the wells were blocked for 30 minutes at 37°C with 0.5% fish skin gelatin (Sigma). After washing three times in PBST, 50 µl/well of supernatant from CHO cells, previously transformed with a plasmid encoding soluble α_d/CD18LZ, was added and the plates incubated at 37°C for 30 minutes. The washing steps were repeated and 50 µl/well hybridoma supernatant was added. Detection of monoclonal antibody was carried out as described above. Positive wells were assayed by flow cytometry using CHO cells transformed with α_d/CD18-encoding DNA as described above and two positive hybridomas designated 212A and 212D were identified. Antibodies secreted by the hybridomas were isotyped as IgG1 using the isotype ELISA procedure described above.

7. In yet another method to generate anti-human α_d monoclonal antibodies, mice were immunized with α_d/CD18LZ Sepharose® beads, prepared as described above, and each mouse receiving 30 µg immunogen on day 0, day 36, and day 66. Mouse #2477 was selected for fusion after screening the mouse sera by the recombinant protein ELISA format as described above. The fusion, selection, and cloning procedures were carried out using the methods described above for fusion 212. Seven positive hybridomas, 217F, 217G, 217H, 217I, 217K, 217L, and 217M were identified, but hybridoma 217F lost reactivity as determined by flow cytometry during the last round of cloning. Antibodies from the six remaining hybridoma lines were isotyped as previously described and all were found to be IgG1.

8. In another method to generate α_d monoclonal antibodies, mouse #2480 was immunized by the same protocol as mouse #2477 but further immunized by interperitoneal injection on days 217 and 218 with 30 µg α_d/CD18LZ. The mouse was sacrificed on day 221, the spleen removed and fusion carried out as described above. Hybridoma supernatant was screened by ELISA as described and flow cytometry to determine reactivity to JY cells previously transfected with DNA encoding α_d/CD18. The screening procedures were carried out as described above. The fusion produced three positive hybridomas 240F, 240G, and 240H, which secreted antibodies isotyped by the

ELISA method to all be IgG1. A fourth hybridoma, 240I, was later characterized to also be an IgG1 isotype.

9. In order to identify antibodies capable of inhibiting functional α_d binding, soluble α_d/CD18LZ (see Example 14) is used for immunization. The protein is isolated on an affinity chromatography resin from supernatant of transiently transfected COS cells and the resin-bound α_d used as an immunogen. A selected mouse is immunized as described above and given a final boost two weeks after the initial immunization. Immunization by this technique prevents possible changes in protein conformation often associated with detergent lysis of cells. Additional mice are immunized with recombinant protein, also resin-bound, but were not initially immunized with protein purified from cell lysate.

Hybridomas, prepared as described above, which result from the immunization are screened by ELISA on the recombinant protein immobilized from a cell supernatant using the Fab fragment of a non-blocking antibody. Alternatively, flow cytomotry is used to assay for reactivity to JY cells previously transfected with α_d cDNA.

10. As another alternative, monoclonal antibodies are generated as follows. Affinity purified α_d/CD18 heterodimeric protein from detergent lysates of stably transfected CHO cells is used with 50 µg/ml muramyl dipeptidase to immunize Balb/c mice as described above. Mice receive three immunizations before serum reactivity against α_d/CD18 is determined by immunoprecipitation of biotinylated complexes in the CHO transfectants. Hybridomas from positive animals are established according to standard protocols, after which hybridoma cultures are selected by flow cytometry using α_d/CD18 transfectants. CD11a/CD18 transfectants are utilized to control for CD18-only reactivity.

11. As another alternative for monoclonal antibody production, Balb/c mice undergo an immunization/immunosuppression protocol designed to reduce reactivity to CHO cell determinants on transfectants used for immunization. This protocol involves immunization with untransfected CHO cells and subsequent killing of CHO-reactive B-cell

blasts with cyclophosphamide treatment. After three rounds of immunization and cyclophosphamide treatment are performed, the mice are immunized with α_d/CD18 CHO transfected cells as described above.

12. As another alternative, CD18 complexes from detergent lysates of PMA stimulated HL60 cells are enriched by preclearance as described above. Other β2 integrins are cleared on the same columns. Immunization with the resulting complexes, hybridoma production, and screening protocols are performed as described *supra*.

Production of Polyclonal Sera

Purified α_d I domain/IgG4 chimera (Example 14) was used to generate polyclonal anti-serum in rabbits. The α_d I domain/IgG4 antigen was injected at 100 μg/rabbit initially in complete Freund's adjuvant, followed by three boosts with the same amount of protein in incomplete Freund's adjuvant. Test bleeds were assayed after the third and fourth injections. Rabbit immunoglobulin (Ig) was purified from the serum on a protein A-Sepharose® column and precleared of anti-human IgG reactivity on a human IgG/Affigel® 10 column. Reactivity by ELISA to the I domain chimera, but not to human IgG, was used to confirm complete preclearance.

The precleared polyclonal sera was used to immunoprecipitate protein from detergent lysates of surface-biotinylated CHO cells previously transfected with α_d and CD18 expression vectors. Immunoprecipitation was carried out by the method previously described in Example 10. The precleared sera recognized a protein complex of the same molecular weight as that precipitated by anti-CD18 monoclonal antibody TS1.18. In addition, the sera recognized a single band of appropriate size in a Western blot of CD18 complexes from α_d/CD18 transfected CHO cells. Affinity purified integrins CD11a/CD18, CD11b/CD18, and VLA4 from human spleen were not recognized by the rabbit polyclonal sera. The sera failed to react with α_d-transfected CHO cells in solution, as determined by flow cytometry. It was therefore concluded that the polyclonal rabbit sera was only capable of recognizing denatured α_d I domain/IgG4 proteins.

In an attempt to produce polyclonal antisera against α_d/CD18, a mouse was immunized 3 times with α_d transfected CHO cells (D6.CHO, α_d/CD18) with adjuvant

peptide and once with purified α_d/CD18 heterodimer. A final boost included only α_d/CD18 heterodimer. Approximately 100 µl immunized serum was precleared by addition of approximately 10^8 LFA-1-transfected CHO cells for 2 hours at 4°C. The resulting serum was assayed for α_d reactivity at dilutions of 1/5000, 1/10000, 1/20000 and 1/40000 on normal human spleen. The polyclonal antibody was reactive at a dilution of 1/20000, while a 1/40000 dilution stained very weakly.

Example 16
Flow Cytometric Analysis Using Anti-α_d Monoclonal Antibodies

Several primary and immortalized cell lines were used in a survey with the anti-α_d monoclonal antibodies 212D, 217K, and 217L. Cell staining was performed and analyzed according to the methods described in Example 17. Primary $CD8^+/CD56^-$ and $CD4^-/CD8^-/CD56^+$ cell lines specific for MAGE-3 (melanoma associated proteins) peptides were strongly positive for CD11b and CD11c, but were not stained by any of the α_d antibodies. MAGE-3 peptide-specific cells are expanded from peripheral blood mononuclear cell populations using peptide-loaded antigen presenting cells (APCs, either dendritic cells or monocytes). Repeated stimulations under limiting dilution conditions, combined with phenotypic selection, result in clonal cytolytic lines which will specifically kill target cells bearing the native protein from which the peptides were derived.

Dendritic cells from peripheral blood, cultured for seven days in the presence of cytokines IL-4 and GM-CSF, were stained strongly by antibodies to CD11a, CD11b, and CD11c, as well as the 217L anti-α_d antibody. The antibodies 212D, 217K, 217I, 217H, and 217M did not react with these cells nor with dendritic cells, obtained from a variety of donors, in repeated experiments. By day 14 of culture, the surface expression of the 217L antigen had waned and the staining disappeared completely by day 21. During the culture period, CD11b and CD11c expression remained at a high level (2 to 3 logs over background staining).

Example 17
Human Monocyte Expression of α_d
Purification of Human Monocytes from Peripheral Blood

Approximately 300 ml of blood was drawn from a volunteer donor into 3.8% sodium citrate buffer (Sigma). The blood was diluted to 480 ml with endotoxin-free PBS (Sigma), and 30 ml of diluted blood was carefully layered onto 17 ml of Histopaque in a 50 ml centrifuge tube. The gradients were spun for 30 minutes at 1500 rpm in a Beckman Tabletop Centrifuge. The cellular layer, representing mononuclear cells, was collected from each gradient and transferred to a new 50 ml tube. The volume was increased to 50 ml with endotoxin-free PBS, 0.1% BSA (endotoxin free), and the tubes centrifuged for 15 minutes at 1500 rpm in a Beckman Tabletop Centrifuge. The supernatant was discarded and the cells resuspended in a small volume of PBS/BSA and subsequently pooled.

A second gradient (which uses Percoll [Denholm and Wolber, *J. Immunol. Meth. 144*:247-251 (1991)]) was required to purify monocytes from the mixed population of cells obtained as described above. Briefly, 10 ml of 10X Hanks buffer (Gibco) was mixed with 600 µl of 1.0 N HCL. To this mixture, 60 ml of Percoll (Pharmacia, Piscataway NJ) was added and the mixture stirred slowly until all Percoll was in solution. The pH of the Percoll solution was adjusted to 7.0, after which 8.0 ml of gradient mixture was added to six 15 ml round-bottomed polystyrene tubes. Exactly 4.0 ml of cell suspension was added to each gradient and the tubes inverted several times to mix thoroughly. The gradients were centrifuged for 25 minutes in a fixed-angle rotor at 1690 rpm at room temperature. The monocyte fraction, which appeared as a thin white band in the gradients, was collected and transferred to new 50 ml centrifuge tubes. The volume was adjusted to 50 ml with PBS/0.1% BSA and the cells pelleted by centrifugation. The cell pellets were resuspended in a small volume and pooled and cell number determined using a hemacytometer. Cells were resuspended in FACS buffer (RPI 1640, 2.0% FBS, 0.2% sodium azide) and adjusted to one million cells/condition, *i.e.*, one million cells were used for each FACS staining condition to assay for various cellular markers.

FACS staining and analysis

Single antibody cell staining was carried out using antibodies immunospecific for α_d or cell markers directly conjugated with a fluorescent tag detectable marker. The mouse anti-human α_d antibodies 212D or 217L were added to cells at 10 µg/ml after which the mixture was incubated on ice for 30 minutes and washed three times. Ten microliters of directly conjugated cell markers, CD3-FITC (Becton-Dickinson) (specific for T cells) or CD33 -FITC (Becton-Dickinson) (specific for monocytes) were added to additional cell samples, while 10 µl of a secondary antibody, anti-mouse FITC (Sigma), was added to the 212D and 217L stained cells. All samples were incubated on ice for 30 minutes in the dark, washed three times, and resuspended in 300 µl of 2.0% paraformaldehyde. Samples were processed on a Becton Dickinson FACScan and the data analyzed using Lysys II software (Becton Dickinson).

In the first experiment, monocytes represented 68% and T-cells 18% of the total cells purified using the double-gradient method. There was a significant amount of staining of both cell types cells for α_d by both 212D and 217L, 55% and 65% of the cells respectively. Based on later experiments, there appeared to be some donor-to-donor variation in the relative amount of α_d staining on freshly isolated human monocytes, although the isolated monocytes always stained positive. When human IgG (used at 1 mg/ml for 10 minutes on ice prior to addition of primary antibody) was added to the cells to block any potential Fc receptor binding problems, there was no change in the α_d staining. When these cells were cultured in suspension using Hydron coated dishes (Interferon Sciences) in 10% FBS/RPM-1640 and analyzed for α_d expression, there was loss of surface expression within 24 hours which continued to diminish over a seven day time course. Relative to expression of other integrins on freshly isolated human monocytes, including CD11a, CD11b, and CD11c, the α_d staining was lower.

2-color FACS staining of Human monocytes for α_d

For 2-color FACS staining, both 212D and 217L antibodies were biotinylated using NHS-LC-biotin (Pierce) according to manufacturer's instruction. In a separate experiment, cells were isolated as described above and stained using biotinylated

212D and 217L antibodies and a biotinylated control IgG1 antibody at 10 μg/ml on ice for 30 minutes. The cells were washed three times in FACS buffer (modified to include D-PBS, 2% FBS, and 0.2% sodium azide), and resuspended in 1.0 ml FACS buffer. Both 10 μl FITC-conjugated CD33 (specific for monocytes) and 5 μl streptavidin PE (PharMingen) were added to cell suspensions. Samples were incubated on ice for 30 minutes in the dark, washed 3 times in FACS buffer, and resuspended in 300 μl 1% paraformaldehyde. Samples were processed by FACS as described above.

Of the two antibodies, 217L showed significant staining on $CD33^+$ cells compared to the control. Antibody 212D also stained this cell type, but the number of $CD33^+$ cells staining was significantly less than observed with antibody 217L. This result was consistent in two separate experiments. In related experiments using biotinylated antibodies 212D and 217L, 217L-biotin consistently stained more cells than 212D-biotin.

Mononuclear cells representing a mixture of lymphocytes and monocytes obtained before Percoll gradient separation were also examined by 2-color analysis as above, and double-stained for 212D and 217L-biotin in combination with FITC-conjugated antibodies immunospecific for CD3 (T cells), CD4 (helper T cells), CD5 (thymocytes, mature T cells, sub-populations of B cells), CD8 (cytotoxic/suppressor T cells), CD14 (monocytes, neutrophils, follicular dendritic reticulum cells), CD20 (B cells), and CD56 (NK cells, subsets of T cells) (Becton Dickinson). No discernible α_d positive populations of cells co-expressed with these cellular markers.

Example 18
Analysis of α_d Distribution

Tissue distribution of α_d/CD18 was determined using polyclonal anti-serum generated as described in Example 15.

Purified rabbit polyclonal antibody was used at concentrations ranging between 120 ng/ml and 60 μg/ml for immunocytochemical analysis of frozen human spleen sections. Sections of 6 micron thickness were layered onto Superfrost Plus Slides (VWR) and stored at -70°C. Prior to use, slides were removed from -70°C and placed at 55°C for 5 minutes. Sections were then fixed in cold acetone for 2 minutes and air dried. Sections were blocked in a solution containing 1% BSA, 30% normal human sera and 5% normal rabbit sera for 30 minutes at room temperature. Primary antibody was

applied to each section for 1 hour at room temperature. Unbound antibody was removed by washing the slides 3 times in TBS buffer for 5 minutes per wash. Next, a rabbit anti-mouse IgG link antibody was applied to each section in the same TBS buffer. A mouse alkaline phosphatase anti-alkaline phosphatase (APAAP) antibody, incubated for 30 minutes at room temperature, was used to detect the second antibody. Slides were then washed 3 times in TBS buffer. Fast Blue substrate (Vector Labs) was applied and color development stopped by immersion in water. Slides were counterstained in Nuclear Fast Red (Sigma) and rinsed in water before mounting with Aqua Mount (Baxter). Staining was detected in the splenic red pulp with this reagent, but not with an irrelevant rabbit polyclonal Ig preparation or the unpurified preimmune serum from the same animal.

Once mouse serum was determined to have specific α_d reactivity, it was used to stain various lymphoid and non-lymphoid tissues. Monoclonal antibodies recognizing CD18, CD11a, CD11b, and CD11c were used in the same experiment as controls. Staining of normal spleen sections with α_d polyclonal sera, and monoclonal antibodies to CD11a, CD11b, CD11c, and CD18 revealed the following results. The pattern observed with α_d polyclonal sera did not display the same pattern of labeling as CD11a, CD11b, CD11c, or CD18. There is a distinct pattern of labeling with some cells located in the marginal zone of the white pulp and a distinct labeling of cells peripheral to the marginal zone. This pattern was not observed with the other antibodies. Individual cells scattered throughout the red pulp were also labeled which may or may not be the same population or subset seen with CD11a and CD18.

Labeling with CD11c did display some cells staining in the marginal zone, but the antibody did not show the distinct ring pattern around the white pulp when compared to α_d polyclonal sera, nor did labeling in the red pulp give the same pattern of staining as α_d polyclonal sera.

Therefore, the labeling pattern seen with α_d polyclonal serum was unique compared to that seen using antibodies to the other β_2 integrins (CD11a, CD11b, CD11c, and CD18), and suggests that the *in vivo* distribution of α_d in man is distinct from that of other β_2 integrins.

Characterization of Human α_d Expression With Monoclonal Antibodies

Antibodies secreted by hybridomas 169A and 169B were used to analyze human α_d expression in frozen tissue sections by immunocytochemistry and on cell lines and peripheral blood leukocytes by flow cytometry. Hybridoma supernatants used in both sets of experiments were undiluted.

Tissue Staining

All stains were carried out as described above, except for liver sections which were stained in the following manner. After acetone fixation, sections were quenched in 1% H_2O_2 and 1% sodium azide in TBS for 15 minutes at room temperature. After primary antibody staining, a rabbit anti-mouse antibody directly conjugated to peroxidase was applied for 30 minutes at room temperature. Slides were washed 3 times in TBS buffer. A swine anti-rabbit antibody, directly conjugated to peroxidase, was incubated for 30 minutes at room temperature to detect the second antibody. Slides were then washed 3 times in TBS buffer and AEC substrate (Vector Labs) was applied and to allow color development. Slides were counterstained with Hematoxylin Gill's No. 2 (Sigma), and subsequently rinsed in water before dehydration and mounting.

In spleen sections, the majority of expression was localized to the splenic red pulp on cells identified by morphology as granulocytes and macrophages. A large number of granulocytes were stained, while only a subset of macrophages gave signal. A small number of follicular dendritic cells in the white pulp also were weakly stained by the α_d antibodies. CD11a and CD18 staining was detected throughout the red and white pulp. CD11c staining was more pronounced in large cells presumed to be macrophages in the splenic white pulp and in the marginal zone surrounding the white pulp; diffuse staining in the red pulp was also noted. CD11b appeared to have distribution overlapping with but not identical to α_d in the red pulp, with no white pulp involvement.

Integrin expression in normal and (rheumatoid) arthritic synovial tissue was compared. Minimal staining with all anti-integrin antibodies (including antibodies specifically immunoreactive with CD11a, CD11b, CD11c, CD18, as well as α_d) was noted in normal tissue, with a widespread distribution on resident cells, presumably macrophages. In the inflamed synovium, expression of all integrins was more localized

to cells clustered around lymphatic vessels. While α_d and CD11b expression patterns were similar, CD11c did not appear to be as strongly expressed and was restricted to a subset of leukocytes.

In the dog, CD11b, but not α_d, expression was observed on liver macrophages, or Kuppfer cells. Staining of normal human liver sections (as previously described for staining of dog liver section, *supra*) confirmed the conservation of this staining pattern in humans. In addition, CD11c was detected at low levels. In sections from a hepatitis patient, all leukointegrin staining was higher than observed on normal liver, while α_d expression was detected on macrophages and granulocytes in these samples.

Minimal staining of normal human colon sections was observed with anti-α_d antibodies; faint smooth muscle staining and leukocyte staining was observed. All leukointegrins were detected at higher levels in sections from patients with Crohn's disease.

Normal lung showed a limited number of weakly α_d-positive cells; these were determined by morphology to be macrophages and neutrophils. In lung tissue from a patent with emphysema, α_d staining was observed on neutrophils and on macrophages containing hemosiderin, an iron-containing pigment, indicating red cell engulfment by these cells.

Sections of normal brain and plaque lesions from patients with multiple sclerosis (MS) were examined for integrin expression. In normal brain, α_d staining was less intense than that of CD11a, CD11b, and CD11c, and restricted to cells typed as microglial cells by morphology and CD68 staining. CD11b positive cells were located surrounding vessels and throughout the tissue. $CD11c^+$ cells appeared to be located within vessels, whereas α_d^+ cells surrounded the vessels. In MS tissue sections, α_d expression was found on both microglial cells and on a non-macrophage leukocyte subset; α_d^+ cells were located within plaque lesions, as well as throughout the cortex. The α_d signal was equivalent in intensity to CD11c, but lower than that of CD11b.

Both thoracic aorta and abdominal aorta sections from PDAY (Pathobiological Determinants of Atherosclerosis in Youth, LSU Medical Center) tissue samples were analyzed with anti-leukointegrin and anti-CAM antibodies. The lesions

examined were consistent with aortic fatty streaks which consisted of subintimal aggregates of large foam cells (mostly macrophages with ingested lipid) and infiltrates of smaller leukocytes. Single label studies with monoclonal antibodies specific for α_d and the other β_2 integrin α chains (CD11a, CD11b, and CD11c), plus a macrophage marker (CD68) revealed that the majority of lipid-laden macrophages expressed a moderate level of α_d and CD18, while expressing CD11a and CD11c at weak or weak to moderate levels, respectively. CD11b was faintly expressed, and then by only a subset of macrophages.

Double label studies were conducted to determine the relative localization of α_d and ICAM-R antigens in the aortic sections. Since foam cells in these sections stained with the antibody Ham 56, specific for a macrophage marker, but not with antibodies to smooth muscle actin, it was determined that the foam cells were not derived from subintimal smooth muscle cells. CD68 positive macrophages expressing α_d were surrounded by and interspersed with small ICAM-R positive leukocytes. There appeared to be a limited number of small leukocytes which were CD68 negative but stained with both α_d and ICAM-R antibodies.

Distribution of α_d in normal tissues appeared to be on resident leukocytes in a pattern overlapping with but not identical to that of CD11b and CD11c, two other leukointegrin α chains which have previously been characterized as having restricted leukocyte distribution. Cellular morphology indicated that α_d staining is largely confined to macrophages and granulocytes, with limited lymphocyte staining. Generally, tissue inflammation appeared to increase the number and types of leukocytes observed in a particular tissue, along with increased staining of leukointegrins, including α_d. Since the cellular and spatial distribution of the leukointegrins was not identical in pathologic tissues, it was inferred that distinct functions and ligands exist for each family member, including α_d, in specific contexts.

Interestingly, α_d expression in early atherosclerotic lesions appeared to be more pronounced than that of CD11a, CD11b, and CD11c, suggesting that α_d may play a central role in the establishment of these lesions. The apposed distribution of α_d and ICAM-R positive cells, supported by evidence suggesting an interaction between α_d and ICAM-R, suggests that α_d may be involved in leukocyte recruitment or activation at early stages in these lesions.

Cell Line and Peripheral Blood Leukocyte Staining

The antibodies 169A and 169B stained a promyeolmonocytic cell line, HL60, by FACS. Surface expression of α_d in these cells is negatively affected by PMA stimulation, which is reported to induce differentiation along a macrophage pathway, but is unaffected by DMSO, which induces granulocyte differentiation [Collins, et al., Blood 70:1233-1244 (1987)]. The FACS profiles of 169A and 169B were antithetical with PMA stimulation to those observed with anti-CD11b and anti-CD11c monoclonal antibodies. A monocyte cell line, THP-1, also exhibited weak staining with 169A and 169B. In addition, a subset of cells in the lymphocyte and monocyte gates of peripheral blood leukocytes appeared to be weakly positive by FACS. A subset of peripheral blood monocytes stained weakly with 169A and 169B, while B lymphocytes were found to have no surface expression of α_d. The $CD8^+$ subset of T lymphocytes was α_d^+. In addition, antibodies 169A and 169B failed to detect antigen on the B cell lines, JY, Ramos, a basophilic line, KU812, and T cell lines, Jurkat, SKW, and Molt 16.

In light of the results with HL60 cells, granulocytes were isolated from peripheral blood by ficoll/hypaque gradient centrifugation and subsequent red blood cells lysis. All preparations were found to be >90% PMNs by visualization of nuclear morphology in acetic acid. Separate populations were stimulated for 30 minutes with 50 ng/ml PMA or 10^{-8} M formyl peptide (fMLP) to release potential intracellular integrin stores. Unstimulated populations exhibited low, but significant expression of 169A and 169B antigens over an IgG1 control, with a detectable increase observed upon stimulation. On PMNs, levels of α_d and CD11c surface expression were more similar than that observed on HL60 cells. The antibody 169B was used subsequently to precipitate a heterodimeric molecule from a detergent lysate of biotinylated PMNs with subunit sizes of approximately 150 and 95 kD appropriate to α_d and CD18, respectively.

The presence of α_d on PMNs could not be anticipated from the information known about canine α_d expression. Canine neutrophils, unlike their human counterparts, express the T helper cell marker CD4, and also integrin VLA-4, and therefore may have different ligands and functions in the dog than in the human.

Staining of PBL subgroups

The present study was undertaken to determine the distribution of this β_2 integrin in human peripheral blood leukocytes. In addition, the cell surface density of α_d relative to other β_2 integrins was compared. Finally, the acute regulation of α_d expression in purified human eosinophils was also evaluated.

Human peripheral blood leukocytes were separated by density gradient centrifugation into a mononuclear cell fraction (containing monocytes, lymphocytes, and basophils) and granulocytes (neutrophils and eosinophils) [Warner, *et al.*, *J. Immunol.Meth.* 105:107-110 (1987)]. For some experiments, eosinophils were purified using CD16 immunomagnetic selection to purities greater than 95% [Hansel, *et al.*, *J.Immunol.Meth.* 122:97-103 (1989)]. Skin mast cells were enzymatically dispersed from human skin and enriched as previously described [Lawrence, *et al.*, *J.Immunol.* 139:3062-3069 (1987)].

Cells were labelled with appropriate dilutions of monoclonal antibody specific for either CD11a (MHM24), CD11b (H5A4), CD11c (BU-15), or α_d (169A). A murine control IgG_1 was also employed. Cells were washed and then incubated with phycoerythrin-conjugated goat-anti-mouse IgG. In some experiments, cells were incubated with excess murine IgG and FITC-labelled murine monoclonal antibody or goat polyclonal antibody specific for a particular cell (*e.g.*, CD3, CD4, or CD8 for T-cells; CD16+ lymphocytes for NK cells; anti-IgE for basophils [Bochner, *et al.*, *J.Immunol.Meth.* 125:265-271 (1989)]. The samples were then examined by flow cytometry (Coulter EPICS Profile) using appropriate gating to identify cell subsets.

For studies with human eosinophils in which acute upregulation of α_d expression was examined, cells were stimulated for 15 minutes at 37°C with phorbol ester (10 ng/ml), RANTES (100 ng/ml) [Schall, *Cytokine* 3:165-183 (1991)], or IL-5 (10 ng/ml) prior to labeling with the various monoclonal antibodies as described above.

Results showed that α_d was present on all peripheral blood eosinophils, basophils, neutrophils, monocytes, and NK cells. A small subset (approximately 30%) of $CD8^+$ lymphocytes was also found to express α_d. Skin mast cells and $CD4^+$ lymphocytes did not express α_d. In general, CD11a and CD11b are present at a higher density on

leukocytes then α_d, the latter being expressed at relatively low levels similar to CD11c. Among leukocytes, monocytes and $CD8^+$ cells have the highest density of α_d, while eosinophils have the lowest level of α_d expression. Expression on neutrophils, basophils, and NK cells was intermediate.

Stimulation of peripheral eosinophils with the CC chemokine RANTES caused no change in the expression of any of the β_2 integrins. Treatment with phorbol ester, however, produced a two to three fold increase in expression of both CD11b and α_d, but did not effect expression of CD11a or CD11c. IL-5 treatment resulted in the selective upregulation of CD11b expression without affecting levels of the other integrin subunits.

Combined, these results indicate that in peripheral blood leukocytes, α_d is generally expressed at a level comparable to CD11c. Highest levels are found on monocytes and a subset of $CD8^+$ lymphocytes. Human skin mast cells do not express α_d. Purified eosinophils appear to have pre-formed intracytoplasmic storage pools of CD11b and α_d. However, the differential upregulation shown by IL-5 versus PMA suggests that these storage pools are separate from each other.

Staining patterns for peripheral blood leukocyte (PBL) subgroups were also determined by flow cytometry using a combination of gating and surface markers, as described above, in an attempt to more precisely define the 169 A/B negative lymphocyte group. PBL were isolated on Ficoll as previously described and stained separately with 169A, 169B and monoclonal antibodies to CD14 (monocyte/macrophage marker), CD20 (B cell), CD56 (NK cell), T cell receptor α/β (T cell), CD16 (neutrophils, NKs), and $\alpha 4$ (a negative marker for neutrophils). Gates were defined by size and marker distribution.

Results indicated that cells in the CD14+ monocyte gate exhibited low levels of 169A and 169B staining. A bimodal expression pattern observed in earlier experiments in the lymphocyte gate was resolved by increasing forward scatter. The mixed $TCR^+/CD20^+$ population appeared to have low, but homogenous levels of 169A/B expression, whereas a population mapped at slightly higher side scatter (cellular complexity), which stained 50% positive for CD56, appeared to have a distinctly 169A/B negative population. The negative population was also not recognized by TCR, CD20, CD14, or CD16 antibodies.

Synovial Distribution of α_d

In order to determine cellular distribution of α_d, other β_2 integrins and their counterreceptors in inflammatory and non-inflammatory synovium, monoclonal antibodies to the various β_2 integrin and immunoglobulin supergene families were used in immunohistological studies. Protein expression was determined in normal, osteoarthritic and rheumatoid synovial tissue samples.

Results indicated that the synovial lining cell layer expressed high levels of VCAM-1, CD11b/CD18 and α_d/CD18. In these cells, CD11c/CD18 expression is restricted and CD11a/CD18 is generally not detected. In rheumatoid arthritis synovitis, expression of β_2 integrins in the synovial cell layer increases in proportion to the degree of hyperplasia. The ratio of cells which express CD11c increases significantly, approaching that of CD11b and α_d, but there is no increase in CD11a expression.

In the sublining areas of the tissue, aggregates and diffuse infiltrates of CD3/CD11a/ICAM-R$^+$ lymphocytes are interspersed among CD68/CD11b/α_d^+ macrophages. A significant number of aggregates demonstrate intense α_d staining, particularly in T cell rich areas.

The synovial endothelium variably expressed ICAM-1 and ICAM-2 with minimal evidence of ICAM-R expression.

Combined, these results indicate that synovial macrophages and macrophage-like synovial cells constitutively express high levels of the β_2 integrins CD11b and α_d. In synovitis, there is an expansion of this subset of cells in both the lining and sublining areas, along with an apparent increase in expression of CD11c. Specific populations of rheumatoid synovial T lymphocytes, in addition to expressing CD11a and ICAM-R, also express high levels of α_d, the latter molecule having been shown above to be expressed at low levels by peripheral blood lymphocytes.

α_d Expression in Disease Lung and Liver Tissue

Lung tissue from an individual with sarcoidosis and liver tissue from two individuals with cirrhosis were sectioned at 6 μm thickness and air dried on Superfrost Plus (VWR Scientific) slides for 15 minutes at room temperature. Prior to use, slides were incubated at 50°C for approximately 5 minutes. Sections were fixed in cold (4°C)

acetone (EM Science) for 2 minutes at room temperature and allowed to air dry at room temperature. Sections were placed in a solution of 100 ml 1X TBS, 1.1 ml 30% H_2O_2 (Sigma), 1.0 ml 10% NaN_3 (Sigma), for 15 minutes at room temperature to remove endogenous peroxidase activity. Each section was blocked with 150 µl of a solution containing 20% normal human serum (Boston Biomedica), 5% normal rat serum (Harlan), and 2% BSA (Sigma) in 1X TBS for 30 minutes at room temperature. After incubation, the solution was gently blotted from the sections. Primary monoclonal antibody was prepared at a protein concentration of 10 µg/ml in blocking solution and 75 µl applied to each tissue section for 1 hour at room temperature. After incubation, sections were washed three times in 1X TBS for 5 minutes each wash to remove unbound antibody. Excess TBS was removed by aspirating around the tissue following the final wash. Biotinylated rat anti-mouse antibody (Jackson Laboratories) was diluted 1:400 in blocking solution and 75 µl was applied to each section for 30 minutes at room temperature. Slides were washed two times with 1X TBS for 5 minutes each wash. Peroxidase conjugated goat anti-biotin antibody (Vector Laboratories) was diluted 1:200 in blocking solution and 75 µl was applied to each section for 30 minutes at room temperature. Slides were washed two times in 1X TBS for 5 minutes each wash. Substrate 3-amino-9-ethylcarbazole (AEC) (Vector Laboratories) or 3,3′-diaminobenzidine (DAB) substrate (Vector Laboratories) was applied and color development stopped by immersion in water. Slides were counterstained in Gill's hematoxylin #2 (Sigma) and rinsed in water before mounting with either Aquamount (Baxter) or Cytoseal (VWR).

In the sarcoidosis lung, only the 217L monoclonal antibody stained cells and the majority of 217L epitope expression was localized to granulomas. Giant cells within the granulomas appeared to be negative for the 217L antigen. Expression of the 217L epitope was localized to cells that morphologically appeared to be epithelioid histiocytes, highly differentiated phagocytic cells of macrophage lineage. Distribution of other integrins was observed to overlap with that of the 217L epitope in the sarcoidosis lung, but the expression patterns were not identical. For example, antibodies immunospecific for all other integrins labeled cells in the granulomas as well as the giant cells which were negative for 217L staining.

Sections from a second patient diagnosed with sarcoidosis were negative for expression of the 217L epitope, however it is unclear from pathology reports whether this patient had received steroidal immunosuppressants, the most common form of treatment.

In sections from the cirrhotic liver tissue, anti-α_d antibodies labeled foam cells in the connective tissue between hepatic nodules as well as a subset of lymphocytes. Distribution of CD11c overlapped with α_d expression but was not identical; anti-CD11c antibody also labeled a subset of foam cells but labeled more macrophages and lymphocytes than anti-α_d antibody. There was no apparent overlap in the distribution of CD11a and CD11b expression with α_d expression.

Antibody 217L also stained phagocytic-type cells which were clustered and isolated from the populations identified by both 212D and 217L. Antibodies to CD11b and CD11c stained the 217L$^+$ clusters in a dissimilar fashion.

In related experiments, antibodies 212D and 217L were used to stain human splenic tissue sections as well as serial sections from spleens of the non-human primate *M. nemestrina*. Splenocytes isolated from fresh human and monkey splenic tissue were also evaluated by flow cytometry for α_d expression. Both antibodies 212D and 217L recognized human and monkey splenocytes. By both ICC and FACS, the α_d^+ population represented about 20% of total cells, unlike in rodents which exhibit a greater percentage of a_d^+ cells. The positive population appeared to be morphologically identical to macrophages.

Human Bone Marrow Staining

Human bone marrow samples were obtained from the iliac bone of healthy bone marrow donors according to standard techniques. The original sample was diluted 1:3 in Iscove's medium and centrifuged for 20 minutes at 2000 RPM. The buffy coat layer was carefully collected, washed once, and hemolyzed using hemolytic buffer (0.83% ammonium chloride, 0.1% sodium bicarbonate, EDTA free). Cells were resuspended in PBS with 15% FBS, aliquoted at 100,000 cells/tube in 100 μl, and put on ice. Immunostaining was performed as previously described. Briefly, monoclonal mouse anti-human α_d antibody 212D or 217L or mouse anti-human CD18 or mouse anti-human

CD50 (ICAM-R specific) antibody was individually added to each cell sample at a final concentration of 10 μg/ml and the mixture incubated on ice for 20 minutes. The cells were washed twice and incubated for an additional 20 minutes with goat anti-mouse FITC. Cells were washed twice and resuspended in 1% paraformaldehyde. Fluorescence was measured using a Fluorescence Activated Cell Sorter FACSCAN (Becton Dickinson).

Results from four experiments indicated that α_d expression as determined using antibody 212D was found on 13 to 43% of the cells (median 27%) and on 6 to 55% of cells (median 21%) using antibody 217L. CD18 expression was observed on 60-96% of cells (median 71%) and CD50 on 86-99% of cells (median 94%).

Expression of α_d on Peripheral Blood
Mononuclear Cells From Patients with Breast Cancer

Peripheral blood mononuclear cells were isolated using Ficoll separation of blood samples from patients with high risk breast cancer, *i.e.*, those patients having breast cancer with poor prognosis features, who had undergone bone marrow transplantation. Cells were screened by immunostaining for the expression of α_d as described above.

Results indicated that α_d expression as determined using antibody 212D was found on 20% of cells and on 13% of the cells using antibody 217L. Antibody 212D also stained a subpopulation of small cells which appeared most likely to be lymphocytes. The percentage of cells expressing α_d were comparable to that generally observed in a normal blood donor.

In addition, antibody 212D appeared to stain not only large cells that were $CD14^+$, but also much smaller cells which were tentatively identified as $CD3^+$. This result was observed both in blood and in bone marrow.

The variation of the number of cells expressing α_d might be explained by a variation in the cell composition of the bone marrow aspirate from donor to donor (e.g., the amount of bone marrow in comparison to the amount of circulating blood).

Example 19
Upregulation of α_d Expression

Because leukocyte integrins are generally upregulated during hemodialysis and contribute to the immune alterations observed in chronic renal failure [Rabb, *et al.*, *J. Am. Soc. Nephrol.* *6*:1445-1450 (1995) and Rabb, *et al.*, *Am. J. Kidnet Dis.* *23*:155-166 (1994)], α_d/CD18 surface expression was examined during hemodialysis and chronic renal failure. In addition, expression of α_d/CD18 *in vitro* following PKC stimulation was also investigated.

Whole blood samples were obtained from five randomly chosen hospital patients with non-renal conditions. Blood samples were incubated with PMA at 50 ng/ml for 30 minutes at 37°C prior to surface staining and flow cytometry. Blood samples were also collected from patients with chronic renal failure. Patients were stable, non-diabetics who were undergoing dialysis three times a week. Baseline samples were obtained prior to beginning dialysis, and subsequent samples were drawn at 15 minutes and 180 minutes during dialysis with a cuprophane membrane. Blood samples from normal subjects having no known diseases were used as negative controls.

For cell staining, 5 µg of antibodies 169A and 169B (and a negative control 1B7) were incubated with 100 µl whole blood in the dark for 15 minutes. Becton Dickinson lysing reagent (2 ml) was added to each mixture and incubation continued for 10 minutes in the dark. Cells were then pelleted and suspended in PBS. The cells were again pelleted by centrifugation and mixed with a secondary FITC-conjugated antibody and incubated for 30 minutes in the dark. Cells were then washed with PBS, centrifuged, aspirated, and resuspended in 1.0% formalin.

Flow cytometry was carried out using the procedure of Rabb, *et al.* [*J. Am. Soc. Nephrol.* *6*:1445-450 (1995)]. Samples were analyzed using Simulset Software (Becton Dickinson) on a FACScan flow cytometer (Becton Dickinson). A minimum of 22,000 cells was analyzed for each sample. Granulocyte, monocyte, and lymphocyte subsets were gated by forward light scatter and side light scatter. Cell subset purity was assessed by CD45 staining and CD14 staining.

Results indicated that α_d/CD18 expression can be detected in samples drawn from normal human subjects; expression was greatest on monocytes and lowest on

lymphocytes. Expression on neutrophils was intermediate between monocytes and lymphocytes. Staining with antibody 169B was weaker than with antibody 169A. PMA treatment upregulated α_d/CD18 expression, particularly on neutrophils and monocytes.

In samples from the renal failure patients, α_d/CD18 expression was detectable on neutrophils, monocytes, and lymphocytes prior to the onset of dialysis. After 15 minutes of dialysis using the leukocyte activating membrane, a minor increase in α_d/CD18 expression was detected. Expression on monocytes and lymphocytes actually decreased by the end of treatment. This result indicates that α_d/CD18 expression is distinct from that observed for CD11a/CD18, CD11b/CD18, and L-selection expression following dialysis.

Example 20
Isolation of Rat cDNA Clones

In view of the existence of both canine and human α_d subunits, attempts were made to isolate homologous genes in other species, including rat (this example) and mouse (Example 20, *infra*).

A partial sequence of a rat cDNA showing homology to the human α_d gene was obtained from a rat splenic λgt10 library (Clontech). The library was plated at 2 x 10^4 pfu/plate onto 150 mm LBM/agar plates. The library was lifted onto Hybond® membranes (Amersham), denatured 3 minutes, neutralized 3 minutes and washed 5 minutes with buffers as described in standard protocols [Sambrook, *et al.*, Molecular Cloning: a laboratory manual, p.2.110]. The membranes were placed immediately into a Stratalinker (Stratagene) and the DNA crosslinked using the autocrosslinking setting. The membranes were prehybridized and hybridized in 30% or 50% formamide, for low and high stringency conditions, respectively. Membranes were initially screened with a ^{32}P-labeled probe generated from the human α_d cDNA, corresponding to bases 500 to 2100 in clone 19A2 (SEQ ID NO: 1). The probe was labeled using Boehringer Mannheim's Random Prime Kit according to manufacturer's suggested protocol. Filters were washed with 2X SSC at 55°C.

Two clones, designated 684.3 and 705.1, were identified which showed sequence homology to human α_d, human CD11b, and human CD11c. Both clones aligned to the human α_d gene in the 3′ region of the gene, starting at base 1871 and extending to

base 3012 for clone 684.3, and bases 1551 to 3367 for clone 705.1.

In order to isolate a more complete rat sequence which included the 5′ region, the same library was rescreened using the same protocol as employed for the initial screening, but using a mouse probe generated from clone A1160 (See Example 20, *infra*). Single, isolated plaques were selected from the second screening and maintained as single clones on LBM/agar plates. Sequencing primers 434FL and 434FR (SEQ ID NOS: 34 and 35, respectively) were used in a standard PCR protocol to generate DNA for sequencing.

5′-TATAGACTGCTGGGTAGTCCCCAC-3′ (SEQ ID NO: 34)

5′-TGAAGATTGGGGGTAAATAACAGA-3′ (SEQ ID NO: 35)

DNA from the PCR was purified using a Quick Spin Column (Qiagen) according to manufacturer's suggested protocol.

Two clones, designated 741.4 and 741.11, were identified which overlapped clones 684.3 and 705.1; in the overlapping regions, clones 741.1 and 741.11 were 100% homologous to clones 684.3 and 705.1. A composite rat cDNA having homology to the human α_d gene is set out in SEQ ID NO: 36; the predicted amino acid sequence is set forth in SEQ ID NO: 37.

Cloning of the 5′ end of Rat α_d

A 5′ cDNA fragment for the rat α_d gene was obtained using a Clonetech rat spleen RACE cloning kit according to manufacturer's suggested protocol. The gene specific oligonucleotides used were designated 741.11#2R and 741.2#1R (SEQ ID NOS: 59 and 58, respectively).

5′-CCAAAGCTGGCTGCATCCTCTC-3′ (SEQ ID NO: 59)

5′-GGCCTTGCAGCTGGACAATG-3′ (SEQ ID NO: 58)

Oligo 741.11#2R encompasses base pairs 131-152 in SEQ ID NO: 36, in the reverse orientation and 741.2#1R encompasses bases pairs 696-715 in SEQ ID NO: 36, also in

the reverse orientation. A primary PCR was carried out using the 3′-most oligo, 741.2#1R. A second PCR followed using oligo 741.11#2R and DNA generated from the primary reaction. A band of approximately 300 base pairs was detected on a 1% agarose gel.

The secondary PCR product was ligated into plasmid pCRTAII (Invitrogen) according to manufacturer's suggested protocol. White (positive) colonies were picked and added to 100 µl LBM containing 1 µl of a 50 mg/ml carbenicillin stock solution and 1 µl M13 K07 phage culture in individual wells in a round bottom 96 well tissue culture plate. The mixture was incubated at 37°C for 30 minutes to one hour. Following the initial incubation period, 100 µl of LBM (containing 1 µl of 50 mg/ml carbenicillin and a 1:250 dilution of a 10 mg/ml kanamycin stock solution) were added and the incubation was continued overnight at 37°C.

Using a sterile 96 well metal transfer prong, supernatant from the 96 well plate was transferred to four Amersham Hybond® nylon filters. The filters were denatured, neutralized and cross linked by standard protocols. The filters were prehybridized in 20 mls of prehybridization buffer (5X SSPE; 5X Denhardts; 1% SDS; 50 ugs/ml denatured salmon sperm DNA) at 50oC for several hours while shaking.

Oligo probes 741.11#1 and 741.11#1R (SEQ ID NOS: 56 and 57, respectively), encompassing base pairs 86-105 (SEQ ID NO: 36) in the forward and reverse orientation respectively, were labeled as follows.

5′-CCTGTCATGGGTCTAACCTG-3′ (SEQ ID NO: 56)
5′-AGGTTAGACCCATGACAGG-3′ (SEQ ID NO: 57)

Approximately 65 WP oligo DNA in 12 µl dH_20 was heated to 65oC for two minutes. Three µl of 10 mCi/ml γ-^{32}P-ATP were added to the tube along with 4 µl 5x Kinase Buffer (Gibco) and 1 µl T4 DNA Kinase (Gibco). The mixture was incubated at 37oC for 30 minutes. Following incubation, 16 µl of each labeled oligo probe were added to the prehybridization buffer and filters and hybridization was continued overnight at 42oC. The filters were washed three times in 5X SSPE; 0.1% SDS for 5 minutes per wash at room temperature, and autoradiographed for 6 hours. Positive clones were expanded and

DNA purified using the Magic Mini Prep Kit (Promega) according to manufacturer's suggested protocol. Clone 2F7 was selected for sequencing and showed 100% homology to clone 741.11 in the overlapping region. The complete rat α_d nucleic acid sequence is set out in SEQ ID NO: 54; the amino acid sequence is set out in SEQ ID NO: 55.

Characteristics of the Rat cDNA and Amino Acid Sequences

Neither nucleic acid nor amino acid sequences have previously been reported for rat α subunits in β_2 integrins. However sequence comparisons to reported human β_2 integrin α subunits suggests that the isolated rat clone and its predicted amino acid sequence are most closely related to α_d nucleotide and amino acid sequences.

At the nucleic acid level, the isolated rat cDNA clone shows 80% identity in comparison to the human α_d cDNA; 68% identity in comparison to human CD11b; 70% identity in comparison to human CD11c; and 65% identity in comparison to mouse CD11b. No significant identity is found in comparison to human CD11a and to mouse CD11a.

At the amino acid level, the predicted rat polypeptide encoded by the isolated cDNA shows 70% identity in comparison to human α_d polypeptide; 28% identity in comparison to human CD11a; 58% identity in comparison to human CD11b; 61% identity in comparison to human CD11c; 28% identity in comparison to mouse CD11a; and 55% identity in comparison to mouse CD11b.

Example 21

Northern Analysis of Rat Tissue For α_d Expression

RNA was obtained from a panel of Lewis rat tissues in order to perform Northern analysis using a rat α_d probe. Samples included total RNA from normal spleen, kidney, liver, lung, and bone marrow, in addition to poly(A^+) RNA from normal spleen, brain, spinal cord, thymus, skin, small intestine, and rat antigen activated T cells and diseased EAE (experimental allergic encephalomyelitis) spleen and lymph node. The experiments were carried out using the techniques described in Example 6.

The α_d probe was selected from a region of the rat cDNA encompassing nucleotides 1184 to 3008 in SEQ ID NO: 54 which represents the area having the lowest degree of homology with rat CD11c and rat CD11b. The 1124 bp probe was generated

by a restriction enzyme digestion with *Eco*R1 of 10 µg rat α_d cDNA clone 684.3. The fragment was gel purified and used in a random primed labelling reaction as described in Example 6. The Northern blot was prehybridized, hybridized and washed as described in Example 6 except the probe was added to the hybridization buffer at 5.5 x 10^5 cpm/ml.

After autoradiography for five days, bands were detected in lanes containing total spleen RNA as well as poly(A^+) RNA from a normal rat as well as a spleen from a rat with active EAE, where the amount of RNA was significantly greater than that from normal spleen. The transcript size detected was consistent with the size of the full length rat cDNA clone.

Example 22
Production and Characterization of Rodent α_d-Specific Antibodies-
<u>Antibodies against Rat α_d I Domain/Hu IgG4 Fusion Proteins</u>

In view of the fact that the I domain of human β_2 integrins has been demonstrated to participate in ligand binding, it was assumed that the same would be true for rat α_d protein. Monoclonal antibodies immunospecific for the rat α_d I domain may therefore be useful in rat models of human disease states wherein α_d binding is implicated.

Oligos "rat alpha-DI5" (SEQ ID NO: 87) and "rat alpha-DI3" (SEQ ID NO: 88) were generated from the rat α_d sequence corresponding to base pairs 469-493 and base pairs 1101-1125 (in the reverse orientation), respectively, in SEQ ID NO: 54. The oligos were used in a standard PCR reaction to generate a rat α_d DNA fragment containing the I domain spanning base pairs 459-1125 in SEQ ID NO: 54. The PCR product was ligated into vector pCRTAII (Invitrogen) according to manufacturer's suggested protocol. A positive colony was selected and expanded for DNA purification using a Qiagen (Chatswoth, GA) Midi Prep kit according to manufacturer's protocol. The DNA was digested with *Xho*I and *Bgl*II in a standard restriction enzyme digest and a 600 base pair band was gel purified which was subsequently ligated into pDCS1/HuIgG4 expression vector. A positive colony was selected, expanded and DNA purified with a Quiagen Maxi Prep Kit.

COS cells were plated at half confluence on 100mm culture dishes and grown overnight at 37oC in 7% CO_2. Cells were rinsed once with 5 ml DMEM. To 5 ml DMEM, 50 µl DEAE-Dextran, 2 µl chloroquine and 15 µg rat α_d I domain/HulgG4 DNA

described above was added. The mixture was added to the COS cells and incubated at 37°C for 3 hours. Media was then removed and 5 ml 10% DMSO in CMF-PBS was added for exactly one minute. The cells were gently rinsed once with DMEM. Ten ml DMEM containing 10% FBS was added to the cells and incubation continued overnight at 37°C in 7% CO_2. The next day, media was replaced with fresh media and incubation continued for three additional days. The media was harvested and fresh media was added to the plate. After three days, the media was collected again and the plates discarded. The procedure was repeated until 2 liters of culture supernatant were collected.

Supernatant collected as described above was loaded onto a ProsepA® column (Bioprocessing Limited) and protein purified as described below.

The column was initially washed with 15 column volumes of Wash Buffer containing 35 mM Tris and 150 mM NaCl, pH 7.5. Supernatant was loaded at a slow rate of less than approximately 60 column volumes per hour. After loading, the column was washed with 15 column volumes of Wash Buffer, 15 column volumes of 0.55 M diethanolamine, pH 8.5, and 15 column volumes 50 mM citric acid, pH 5.0. Protein was eluted with 50 mM citric acid, pH 3.0. Protein was neutralized with 1.0 M Tris, pH 8.0, and dialyzed in sterile PBS.

The rat α_d I domain protein was analyzed as described in Example 14. The detected protein migrated in the same manner as observed with human I domain protein.

Production of Monoclonal Antibodies to Rat α_d I Domain/HuIgG4 Fusion Proteins

Mice were individually immunized with 50 µg purified rat α_d I domain/HulgG4 fusion protein previously emulsified in an equal volume of Freunds Complete Adjuvant (FCA) (Sigma). Approximately 200 µl of the antigen/adjuvant preparation was injected at 4 sites in the back and flanks of each of the mice. Two weeks later the mice were boosted with an injection of 100 µl rat α_d I domain/HuIgG4 antigen (50 µg/mouse) previously emulsified in an equal volume of Freunds Incomplete Adjuvant (FIA). After two additional weeks, the mice were boosted with 50 µg antigen in 200 µl PBS injected intravenously.

To evaluate serum titers in the immunized mice, retro-orbital bleeds were performed on the animals ten days following the third immunization. The blood was

allowed to clot and serum isolated by centrifugation. The serum was used in an immunoprecipitation on biotinylated (BIP) rat splenocytes. Serum from each mouse immunoprecipitated protein bands of expected molecular weight for rat α_d and rat CD18. One mouse was selected for the fusion and was boosted a fourth time as described above for the third boost.

The hybridoma supernatants were screened by antibody capture, described as follows. Immulon® 4 plates (Dynatech, Cambridge, Massachusetts) were coated at 4°C with 50 µl/well goat anti-mouse IgA, IgG or IgM (Organon Teknika) diluted 1:5000 in 50 mM carbonate buffer, pH 9.6. Plates were washed 3X with PBS containing 0.05% Tween® 20 (PBST) and 50 µl culture supernatant was added. After incubation at 37°C for 30 minutes, and washing as described above, 50 µl horseradish peroxidase-conjugated goat anti-mouse IgG9(Fc) (Jackson ImmunoResearch, West Grove, Pennsylvania) diluted 1:3500 in PBST was added. Plates were incubated as described above and washed 4X with PBST. Immediately thereafter, 100µl substrate, containing 1 mg/ml *o*-phenylene diamine (Sigma) and 0.1 µl/ml 30% H_2O_2 in 100 mM citrate, pH4.5, was added. The color reaction was stopped after 5 minutes with the addition of 50 µl 15% H_2SO_4. Absorbance at 490 nm was read on a Dynatech plate reader.

Supernatant from antibody-containing wells was also analyzed by ELISA with immobilized rat α_d I domain/HulgG4 fusion protein. An ELISA with HuIgG4 antibody coated plates served as a control for reactivity against the IgG fusion partner. Positive wells were selected for further screening by BIP on rat splenocyte lysates using techniques described below.

<u>Production of Polyclonal Sera To Rat α_d I domain/HuIgG4 Fusion Protein</u>

Two rabbits were prebled prior to immunization with 100 μg purified rat α_d I domain/HuIgG4 fusion protein in complete Freund's adjuvant. Injections were repeated at the same dose every three weeks in incomplete Freunds adjuvant (IFA). After three injections the rabbits were test bled and the collected sera used in a standard immunoprecipitation on rat splenocyte lysates. It was determined that sera from both rabbits were immunoreactive with rat α_d. The rabbits were boosted again with 100 ug antigen in IFA, and the collected sera assayed for increased immunoreactivity with rat α_d

by immunoprecipitation. The animals were given a final boost and 10 days later, bled out and sera collected.

Rat α_d Histology

Rabbit polyclonal sera generated against rat α_d "I" domain was used in immunohistochemical staining of rat tissue sections by the technique described in Example 18. The staining pattern detected on frozen and on paraffin embedded rat spleen sections was essentially identical to that observed with the antibodies against human α_d, with staining individual cells throughout the red pulp. The staining pattern differed from that observed with monoclonal antibodies against rat CD11a, CD11b and CD18. In addition, a positive staining pattern was seen in the thymus on individual cells throughout the cortex. Neither of these tissue gave any signal when stained with the rabbit preimmune sera.

Analysis of Antibody Specificity

Rats were sacrificed by asphyxiation with CO_2 and spleens were removed using standard surgical techniques. Splenocytes were harvested by gently pushing the spleen through a wire mesh with a 3 cc syringe plunger in 20 mls RPMI. Cells were collected into a 50 ml conical tube and washed in the appropriate buffer.

Cells were washed three times in cold D-PBS and resuspended at a density of 10^8 to 10^9 cells in 40 ml PBS. Four mg of NHS-Biotin (Pierce) was added to the cell suspension and the reaction was allowed to continue for exactly 15 minutes at room temperature. The cells were pelleted and washed three times in cold D-PBS.

Cells were resuspended at a density of 10^8 cells/ml in cold lysis Buffer (1% NP40; 50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 2 mM CaCl; 2 mM MgCl; 1:100 solution of pepstain, leupeptine, and aprotinin, added just before adding to cells; and 0.0001 g PMSF crystals, added just before adding to cells). Lysates were vortexed for approximately 30 seconds, incubated for 5 minute at room temperature, and further incubated for 15 minutes on ice. Lysates were centrifuged for 10 minutes at 10,000 xg to pellet the insoluble material. Supernatant was collected into a new tube and stored at between 4°C and -20oC.

One ml cell lysate was precleared by incubation with 200 μl of a protein A Sepharose® slurry (Zymed) overnight at 4°C. Precleared lysate was aliquoted into Eppendorf tubes at 50 μl/tube for each antibody to be tested. Twenty-five μl of polyclonal serum or 100 to 500 μl of monoclonal antibody supernatant were added to the precleared lysates and the resulting mixture incubated for 2 hours at 4°C with rotation. One hundred μl rabbit anti-mouse IgG (Jackson) bound to protein A Sepharose® beads in a PBS slurry was then added and incubation continued for 30 minutes at room temperature with rotation. Beads were pelleted with gentle centrifugation, and washed three times with cold Wash Buffer (10 mM HEPES; 0.2 M NaCl; 1% Trition X-100). Supernatant was removed by aspiration, and 20 μl 2X SDS sample buffer containing 10% β-mercaptoethanol was added. The sample was boiled for 2 minutes in a water bath, and the sample loaded onto a 5% SDS PAGE gel. Following separation, the proteins were transferred to nitrocellulose at constant current overnight. The nitrocellulose filters were blocked with 3% BSA in TBS-T for 1 hour at room temperature and the blocking buffer was removed. A 1:6000 dilution of Strepavidin-HRP conjugate (Jackson) in 0.1% BSA TBS-T was added and incubation continued for 30 minutes at room temperature. Filters were washed three times for 15 minutes each with TBS-T and autoradiographed using Amersham's ECL kit according to manufacturer's suggested protocol.

<u>Production of Monoclonal Antibodies To Full Length Rat α_d Protein</u>

Rat α_d was purified from rat splenocytes to prepare an immunogen for generating anti-rat α_d monoclonal antibodies. Spleens from approximately 50 normal female Lewis rats, 12-20 weeks of age, were collected and a single cell suspension was made from the tissue by forcing it through a fine wire screen. Red blood cells were removed by lysis in buffer containing 150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4, and remaining leukocytes were washed two times with phosphate buffered saline (PBS). The splenocytes were pelleted by centrifugation and lysed in buffer containing 50 mM Tris, 150 mM NaCl, 2 mM CaCl2, 2mM MgCl2, 10 mM PMSF, leupeptin, pepstatin and 1% Triton X-100®. Splenocyte lysis was carried out on ice for 30 minutes with one ml of lysis buffer per 5 x 10^8 splenocytes. Insoluble material was removed by centrifugation.

CD11a, CD11b and CD11c were removed from the spleen lysate by immunoprecipitation as follows. A 750 µl volume of a Protein A-Sepharose® slurry was incubated with 2 mg rabbit anti-mouse immunoglobulin at 4°C for 30 minutes. The rabbit anti-mouse-Protein A-Sepharose® was washed three times with lysis buffer and suspended in a final volume of 1.5 ml of lysis buffer. Approximately 200 µg each of rat β_2 integrin specific monoclonal antibodies, 515F (specific for rat CD11a), OX-42 (specific for rat CD11b) and 100g (specific for rat CD11c) were each added to 50 ml of the rat spleen lysate. Following a 30 minute incubation at 4°C, 500 µl of the rabbit anti-mouse-Protein A-Sepharose® was added to the spleen lysates and mixed with end-over-end rotation for 30 minutes at 4°C. The lysate was centrifuged at 2500 x *g* for 10 minutes to pellet the CD11a, CD11b, and CD11c bound to the rabbit anti-mouse-Protein A-Sepharose®, and the supernatant transferred to a clean 50 ml centrifuge tube. Immunoprecipitation with the antibodies 515F, OX-42, and 100g was repeated two additional times to insure complete removal of CD11a, CD11b, and CD11c.

β_2 integrins remaining in the lysate were isolated using affinity purification. Approximately 250 µl of a slurry of anti-rat CD18 monoclonal antibody 20C5B conjugated to CNBr-Sepharose® was added to the lysates and mixed with end-over-end rotation for 30 minutes at 4°C. Antibody/antigen complexes were pelleted by centrifugation at 2500 x *g* for ten minutes and the pellet washed three times with lysis buffer before being stored at 4°C.

Immunization of Armenian Hamsters

1. Armenian hamsters, six to eight weeks old, were initially immunized with approximately 50 µg of a recombinant protein consisting of the I domain of rat α_d fused to the human IgG_4 heavy chain emulsified in complete Freund's adjuvant. Primary immunization was followed by subsequent immunizations with rat α_d I domain/$HuIgG_4$ emulsified in incomplete Freund's adjuvant on Days 14, 33, and 95. Two separate fusions, designated 197 and 199, were subsequently performed.

Four days prior to fusion 197 (day 306), one hamster was administered a combination of rat α_d protein purified from splenocytes and CHO cells transfected with rat α_d. The fusion boost was given three days prior to the fusion (day 307) with purified

rat α_d protein and α_d transfected CHO cells. Rat α_d transfected CHO cells were prepared as described below.

A gene segment encoding full length rat α_d protein was inserted into the pDC1 vector and transfected by electroporation into CHO cells together with a human CD18-pRC construct. Transfected cells were grown in the presence of hypoxanthine to select for cells successfully transfected with the pRC construct and in the presence of g418 to select for cells transfected with the pDC1 construct. After 3 weeks, the cells were stained with the rat α_d specific rabbit polyclonal sera and sorted by FACS. A small percentage of the cells which expressed the highest levels of surface α_d (approximately 3% of the total population) were collected and further expanded. FACS selection was repeated several times to provide a population cells with high levels of α_d surface expression.

The α_d transfected cells were also characterized by flow cytometry using a rat α_d specific polyclonal sera and a human CD18 specific monoclonal antibody, TS1.18.1. Results confirmed that the transfected CHO cells expressed high levels of both rat α_d and human CD18.

Finally, α_d and CD18 expression in the cells was evaluated by immunoprecipitation. A rat α_d specific rabbit polyclonal sera was found to immunoprecipitate proteins with two distinct molecular weights: the higher molecular weight protein(s) being approximately 170 kD, and the lower molecular weight protein(s) 95 kD. These findings were consistent with expression of a rat α_d/human CD18 heterodimeric complex on the surface of the transfected CHO cells.

On the day of the fusion, the spleen was removed and a single-cell suspension was formed by grinding the tissue between frosted ends of two glass microscope slides submerged in serum free RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 µg/ml streptomycin (RPMI) (Gibco, Canada). The cell suspension was filtered through sterile 70-mesh Nitex cell strainer (Becton Dickinson, Parsippany, New Jersey), and washed twice by centrifuging at 200 x *g* for five minutes and resuspending the pellet in 20 ml serum free RPMI. Thymocytes taken from three naive Balb/c mice were prepared in a similar manner. NS-1 myeloma cells, kept in log phase in RPMI with 10% Fetaclone serum

(FBS) (Hyclone Laboratories, Inc. Logan, Utah) for three days prior to fusion, were centrifuged at 200 x *g* for five minutes, and the pellet was washed twice as previously described.

Approximately 1.15 x 10^8 spleen cells were combined with 5.8 x 10^7 NS-1 cells, centrifuged and the supernatant removed by aspiration. The cell pellet was dislodged by tapping the tube and seven ml of 37°C PEG 1500 (50% in 75 mM Hepes, pH 8.0) (Boehringer Mannheim) was added with stirring over the course of one minute, followed by adding 14 ml of serum free RPMI over seven minutes. An additional eight ml RPMI was added and the cells were centrifuged at 200 x *g* for 10 minutes. The supernatant was removed and the pellet resuspended in 200 ml RPMI containing 15% FBS, 100 mM sodium hypoxanthine, 0.4 mM aminopterin, 16 mM thymidine (HAT) (Gibco), 25 units/ml IL-6 (Boehringer Mannheim) and 1.5 x 10^6 thymocytes/ml. The suspension was dispensed into ten 96-well flat bottom tissue culture plates (Corning, United Kingdom) at 200 µl/well and the cells were fed on days 4, 5, 6, and 7 days post fusion by aspirating approximately 100 µl from each well with an 18 G needle (Becton Dickinson) and adding 100 µl plating medium described above except lacking thymocytes.

On day 10, supernatants from the fusion wells were screened by flow cytometry for reactivity to rat α_d/human CD18 transfected CHO cells. Approximately 5 x 10^5 rat α_d transfected CHO cells were suspended in 50 µl RPMI containing 2.0% FBS and 0.05% sodium azide and added to approximately 100 µl of hybridoma culture supernatant in 96-well, round-bottomed plates. Positive controls for staining included rabbit anti-α_d polyclonal sera and TS1/18 (anti-human CD18). Cells were incubated for 30 minutes on ice, washed three times in FACS buffer (RPMI, 2.0% FBS, 0.05% NaAzide), and incubated for 30 minutes on ice with a FITC-conjugated goat anti-hamster antibody (Jackson ImmunolResearch Labs) at a final dilution of 1:200 in FACS buffer. Cells were washed three times in FACS buffer and resuspended in 200 ml of FACS buffer. Samples were analyzed with a Becton Dickinson FACscan analyzer. To insure that positive clone wells were specific for rat α_d, the screen was repeated with non-transfected CHO cells. Wells which met the criteria of reacting with rat α_d CHO transfectants and not the untransfected CHO cells were cloned.

Following primary screening, cells from positive wells were cloned initially

by doubling dilution and subsequently by limiting dilution in RPMI, 15% FBS 100 mM sodium hypoxanthine, 16 mM thymidine, and 10 units/ml IL-6. In the limiting dilution step, the percentage of wells showing growth was determined and clonality was predicted using a Poisson distribution analysis. Wells showing growth were analyzed by FACS after 10-12 days. After final cloning, positive wells were expanded in RPMI and 11% FBS. Cloning yielded one culture deemed positive by these criteria, from which four separate subclones designated 197A-1, 197A-2, 197A-3, and 197A-4 were expanded.

Prior to fusion 199, a second hamster was boosted on day 307 with 2.3 x 10^6 rat α_d (RAD)-transfected CHO cells. Two final immunizations were administered four days prior to the fusion (day 334) and again three days prior to the fusion (day 335). The boost on day 334 consisted of 2 x 10^6 rat α_d transfected CHO cells and 200 µl of purified rat α_d bound to Sepharose® (described previously) administered by intraperitoneal injection. The day 335 boost consisted of 5 x 10^6 rat α_d transfected CHO cells, also administered by intraperitoneal injection. The fusion and screening protocols for fusion 199 were identical to fusion 197, and three hybridomas, designated 199A, 199H, and 199M, with supernatant reactive with rat α_d were identified and cloned.

2. A second immunization was carried out using the same protocols which led up to fusions 197 and 199. After the day 334 boost, there were no further immunizations until days 394 and 395. Prior to the fusion, the hamsters were administered 2 x 10^6 RAD-transfected CHO cells along with 300 µl of purified rat α_d-Sepharose® which was administered interpertoneally. The fusion and screening protocols for the subsequent fusion 205 were identical to those of fusions 199 and 197, except that during cloning, Armenian hamster ELISA reagents, e.g. goat anti-Armenian hamster antibodies (Jackson ImmunolResearch Labs), were used as an initial screen. Positive wells identified by this method were subsequently screened by FACS as described. Fusion 205 yielded three separate positive clones named 205A, 205C, 205E.

3. In another method to generate anti-rat α_d monoclonal antibodies, 6 to 12 week old BALB/c mice were immunized on day 1 with purified rat α_d-Sepharose® administered subcutaneously in complete Fruend's adjuvant. A second boost was

administered by the same route on day 25 with the same immunogen in incomplete Freund's adjuvant. A third boost identical to the second was performed on day 42. No further boosts were carried out until the pre-fusion boosts which consisted of 400 µl (for fusion 226) and 250 µl (for fusion 236) purified rat α_d-Sepharose® injected intraperitoneally. Each volume contained approximately 10 to 15 µg antigen as determined by Coomassie staining. The prefusion boosts for fusion 226 occurred on days 62 and 63 and the fusion was performed on day 66. For fusion 236, the prefusion boosts were performed on days 132 and 133 and the fusion was performed on day 136. Both fusion protocols differed from that used for the Armenian hamster fusions described above in that a 5:1 ratio of splenocytes to NS-1 cells was used as compared to a ratio of 2:1 in the Armenian hamster fusions. The fusion protocol was otherwise identical to the Armenian hamster protocol.

The screening and cloning protocols for fusions 226 and 236 were identical to those used in fusions 197, 199, and 205, except that an initial screen by ELISA was performed. In the ELISA, a goat anti-mouse whole molecule was employed to capture the mouse antibody from hybridoma supernatant and a goat anti-mouse horse radish peroxidase conjugate was used to detect mouse antibody. Positive supernatants were subsequently screened by FACS as described for fusions 197 through 205.

Fusion 226 yielded nine positive clones designated 226A, 226B, 226C, 226D, 226E, 226F, 226G, 226H, and 226I. Fusion 236 yielded ten positive clones designated 236A, 236B, 236C, 236F, 236G, 236H, 236I, 236K, 236L, and 236M. Monoclonal antibodies generated from these clones were isotyped by ELISA as described in Example 15. All antibodies were found to be of the IgG1 isotype.

Characterization of Monoclonal Antibodies to Rat α_d

In order to characterize the anti-rat α_d antibodies, biotin labeled spleens lysates were prepared as described in Example 22, section D, above. Lysates were precleared prior to use in immunoprecipitations. Initially, 50 µg/ml of normal murine immunoglobulin was added to the lysate and the resulting solution mixed with end-over-end rotation for 30 minutes at 4°C. A 75 µl slurry of a protein A-Sepharose® coated with rabbit anti-mouse immunoglobulin was added and mixing was continued with end-over-

end rotation for 30 minutes. The rabbit anti-mouse coated protein A beads were pelleted by centrifugation at 15,000 rpm in a table-top microfuge for five minutes at 4°C and the supernatant collected. The pelleted material was discarded.

For each cloned hybridoma, approximately 300 µl of supernatant was placed into a Eppendorf microfuge tube, to which was added 30 µl 10% Triton X-100®, 30 µl of a 100X stock solution of pepstatin, leupeptin and aprotinin, 100 µg PMSF crystals, and 50 µl of precleared biotinylated rat spleen lysate. Samples were vortexed gently and placed onto an end-over-end rotator at 4°C for 30 minutes. A control sample was prepared by adding 10 mg/ml of a rabbit anti-rat α_d specific polyclonal antibody to 50 µl of rat spleen lysate.

Following a 30 minute incubation, 75 µl of protein A-Sepharose® beads in a PBS slurry was added to each sample and incubated with end-over-end rotation at 4°C for 30 minutes. The protein A-coupled beads were pelleted by centrifugation at 15,000 rpm in a table-top microfuge for 5 minutes at 4°C and the supernatant was collected. The pelleted beads were washed sequentially with a series of 1 ml detergent washes as follows: buffer #1 containing 10mM Tris, 400 mM NaCl, 1.0% Triton X-100®, pH 8.0; buffer #2 containing 10mM Tris, 400 mM NaCl, 0.5% Triton X-100®, pH 8.0; buffer #3 containing 10mM Tris, 400 mM NaCl, 1.0% Triton X-100®, 0.1% deoxycholate, pH 8.0; and buffer #4 containing 10 mM Tris, 400 mM NaCl, 0.5 M $LiCl_2$, pH 8.0. A final washed was carried out with wash buffer #1. Beads were vortexed gently between each wash and pelleted using a tabletop microfuge. Supernatants were removed by transfer pipette, and after the final wash, all remaining buffer was removed from the beads by Hamilton syringe. A 50 µl aliquot of SDS sample buffer containing Bromphenol Blue and Pyronine Y dyes and β-mercaptoethanol at a final concentration of 10% was added to each pellet. The mixture was vortexed vigorously for 1-2 minutes and incubated at room temperature for 5-10 minutes. Samples were centrifuged for 5 minutes at 15,000 rpm in a table-top microfuge at 4°C and released protein was collected and transferred to a new microfuge tube. Aliquots from each sample were boiled for four minutes in a water bath before loading onto 7.5% SDS-PAGE gels. Following separation by PAGE, proteins were transferred to nitrocellulose filters for one hour at 200 mAmps, and the filters were blocked in a solution of 3.0% BSA/TBS-T overnight at 4°C. A solution of

0.1% BSA-TBS-T containing a 1:6000 dilution of streptavidin-OPD was added to each filter and incubation allowed to continue for one hour at room temperature. The filters were washed five times for ten minutes each in TBS-T, and developed using Amersham's ECL kit according to the manufacturer's suggested protocol.

Clone 199M was found to immunoprecipitate a heterodimeric protein. The larger protein subunit had an approximate molecular weight of 170-175 kD which was consistent with the size of the protein immunoprecipitated by the rabbit anti-rat α_d polyclonal control. A second protein was also precipitated with an approximate molecular weight of 95 kD, consistent with the weight of CD18.

Example 23

Specificity of Monoclonal Antibody 199M

A CNBr-Sepharose® affinity column with conjugated 199M monoclonal antibody was used to affinity purify rat α_d from spleen cell lysates. Briefly, approximately 1.3×10^{10} rat spleen cells were lysed in buffer consisting of 150 mM NaCl, 10 mM PMSF, 10 mM Tris, 1% Triton X-100®, pH 8.0. Cells in the buffer were incubated for 30 minutes on ice and centrifuged at approximately 10,000 x *g* for 30 minutes at 4°C.

Antibody 199M was conjugated to CNBr-activated Sepharose® 4B (Pharmacia) by the following method. One gram of the activated resin was suspended in 1 mM HCl for 15 minutes and washed three times with 15 ml of 1 mM HCl and once with 15 ml coupling buffer containing 0.1 mM HCO_3, 0.5 M NaCl, pH 8.0. Antibody 199M in coupling buffer was added to resin suspension at a final concentration of approximately 10-20 mg/ml and the mixture incubated overnight at 4°C. The following day the conjugated resin was pelleted by centrifugation and the supernatant removed. Unreacted groups on the resin were blocked by incubation in 0.1 M Tris, pH 8.0 for one hour at room temperature. The conjugated resin was washed with 0.1 M citric acid, pH 3.0, and stored in lysis buffer as a 1:2 slurry containing 0.1 % sodium azide.

For affinity purification, spleen cells were incubated with end-over-end mixing overnight with 0.4 ml of the 199M-conjugated Sepharose® resin. The resin was then pelleted by centrifugation and washed four times with 15 ml lysis buffer. Aliquots of approximately 100 µl of each gel were boiled briefly in reducing sample buffer containing 0.1 M Tris-HCl, pH 6.8, 2.0% SDS, 20% glycerol, 0.0002% bromophenol

blue, 10% β-mercaptoethanol (final concentration 5%) and loaded onto and proteins resolved using a 6.0% polyacrylamide SDS gel (SDS-PAGE).

The affinity purified material was found to contain two major and one minor protein species when separated on SDS-PAGE. A prominent protein band with a molecular weight of 90 kD was consistent with the known size of CD18 and this band was not sequenced. A second prominent band of 160 kD was detected which was consistent with the predicted molecular weight for α_d. In addition, a minor band with an apparent molecular weight of 200 kD was also detected. Both the 160 kD and 200 kD species were further analyzed by amino terminal protein sequencing with the results compared to the amino acid sequence predicted by the rat α_d cDNA, as well as to the known amino acid sequences for CD11c and CD11b. The sequence of both the 160 and 200 kD bands were found to be consistent with the amino acid sequence predicted by cloned rat α_d, suggesting that there may be two forms of α_d, perhaps resulting from splice variants or glycosylation differences.

Example 24

T Cell Proliferation Assay Using Rat α_d-Expressing Macrophages

Macrophages expressing α_d isolated from rat spleens were used as antigen presenting cells (APC) to stimulate a myelin basic protein specific T cell line designated LR-21. Briefly, rats were injected intravenously with a 100 µl volume of iron particles (BioMag, Cambridge, MA). The following day a single cell suspension was prepared from the spleens and α_d^+ macrophages which had phagocytosed iron particles were collected using a magnet. Flow cytomotery and immunoprecipitation indicated that 50 to 80% of the cells which phagocytose iron are α_d^+.

The results indicated that spleen macrophages expressing α_d were very poor APC's compared to other APC such as thymic macrophages. The monoclonal antibody designated 205C was also tested in the proliferation assay with the α_d positive macrophages and the LR-21 cell line. Proliferation assays were then carried out as follows.

Spleen macrophages positive for α_d expression were suspended at a density of 6 x 10^6 cells/ml in RPMI containing 5% normal rat serum and 100 µl of the macrophage suspension was added to each well. Cells from the LR-21 line were

suspended at 1 x 10^6 cell/ml in RPMI with 5% normal rat serum and 50 µl of the suspension was added to each well. Monoclonal antibody 205C was added to each well in a volume of 50 µl to a final concentration of 50, 10 and 2 µg/ml. Plates were incubated at 37°C for 72 hours and 1 µCi ^{3}H-thymidine was added for the final 24 hours of incubation. Cells were harvested onto glass fiber mats and ^{3}H incorporation determined using a Direct Beta Counter (Packard Matrix 96).

Results from the experiments indicate that high concentrations of antibody 205C (10 and 50 µg/ml) are able to reduce T cell proliferation in a dose dependent manner.

Example 25
Immunoprecipitation of α_d from Rat Bone Marrow

Bone marrow cells were harvested from a Lewis rat by flushing the femur bone with PBS. The cells were washed, biotinylated, and immunoprecipitated, essentially as described in Example 18, using 20 µg purified monoclonal antibodies to immunoprecipitate protein from 100 µl of precleared cell lysates. Detection of immunoprecipitated protein was carried out in the manner as previously described.

The rat α_d monoclonal antibody 205C immunoprecipitated two bands which migrated at 160 kD and 95 kD. Bands of this size were consistent with the size for the α and β chains in α_d/CD18 as observed in immunoprecipitation of proteins from spleen cell lysates using the same antibody. Antibodies against rat CD11a, CD11b, or CD11c immunoprecipitated alpha chains distinct from α_d and all antibodies co-immunoprecipitated a protein having a molecular weight consistent with that known for CD18.

Example 26
Expression of α_d in Animal Models

Preliminary results indicated that rat α_d is selectively expressed by subpopulations of macrophages, including cortical macrophages in the thymus, Kupffer cells in the liver, perivascular cells in the central nervous system, a subset of peritoneal macrophages, and resident bone marrow macrophages. In addition, a subset of thioglycolate macrophages showed upregulation of α_d expression following stimulation

with dexamethasone. The observed macrophage-restricted expression of rat α_d suggested further analysis of expression in various animal models.

Expression of Rat α_d in Phenylhydrazine Model

The administration of phenylhydrazine to animals results in massive red blood cell (rbc) damage which leads to a transient anemia. Damaged rbcs are cleared from circulation by red pulp macrophages, resulting in significant splenomegaly. It is proposed that macrophages which express α_d may be involved in the clearance of damaged rbcs and other foreign material from circulation.

To test this hypothesis, groups of rats were treated with saline alone or phenylhydrazine dissolved in saline and administrated by intraperitoneal injection at a dosage of 100 mg/kg body weight. In some experiments rats were treated with a polyclonal antiserum generated to the "I domain" of rat α_d.

At various time points following phenylhydrazine administration, animals were sacrificed. Spleen weight and hematocrit were used as parameters of rbcs clearance. In addition, kidney, spleen and liver were collected for histopathologic evaluation, which included immunostaining for CD11a, CD11b, CD11c and α_d.

Gross findings indicated that four days following treatment with phenylhydrazine (saline controls and α_d treatment) rats developed a dramatic splenomegaly, while hematocrit levels dropped. Treatment with the α_d polyclonal serum had no effect on spleen weight or the drop in hematocrit induced with phenylhydrazine.

Tissue from saline and day 4 phenylhydrazine treated rats were sectioned at 4 µm thickness and air dried on Superfrost Plus (VWR Scientific) slides at room temperature for 15 minutes. Prior to use, slides were incubated at 50°C for approximately 5 minutes. Sections were fixed in cold (4°C) acetone (EM Science) for 2 minutes at room temperature and allowed to dry at room temperature. Sections were placed in 100 ml 1X TBS, 1.1 ml 30% H_2O_2 (Sigma), 1 ml 10% NaN_3 (Sigma) for 15 minutes at room temperature to remove endogenous peroxidase activity. Each section was blocked using 150 µl of a solution containing 30% normal rat serum (Harlan Bioproducts), 2% BSA (Sigma) in 1X TBS for 30 minutes at room temperature, after which the solution was gently blotted from the sections. Each section received 75 µl of biotinylated hamster anti-

rat α_d antibody 205C at a protein concentration of 13.3 µg/ml diluted in blocking solution, for 1 hour at room temperature. After incubation, the sections were washed three times in 1X TBS for 5 minutes each to remove any unbound antibody. Excess TBS was removed by aspirating around the tissue following the final wash. Peroxidase-conjugated goat anti-biotin antibody (Vector Laboratories) was diluted 1:200 in blocking solution and 75 µl was applied to each section for 30 minutes at room temperature. After incubation, slides were washed two times in 1X TBS for 5 minutes each wash. AEC substrate (Vector Laboratories) was applied and color development stopped by immersion in water. Slides were counterstained in Gill's hematoxylin #2 (Sigma) and rinsed in water, after which they were successively dehydrated in 70%, 95%, 100% EtOH, and Xylene. Sections were then mounted with cytoseal (VWR).

In the saline-treated rat spleen sections, the majority of α_d expression was localized in the splenic red pulp on cells identified morphologically as macrophages, granulocytes, and a subpopulation of lymphocytes. In the phenylhydrazine-treated rat spleens, however, the splenic red pulp had undergone morphological changes such that the only cell type identified was a population of large macrophages which had engulfed damaged red blood cells. The majority of these large macrophages were observed to expressed α_d. Also in the phenylhydrazine-treated rat, there appeared to be an increase in the number of macrophages in the splenic white pulp that expressed α_d.

A double label experiment to determine expression of α_d and CD11c was also performed on a phenylhydrazine-treated rat spleen. As previously described, α_d expression was detected on large macrophages in the splenic red pulp that appeared to have engulfed damaged red blood cells. CD11c expression was also detected on large macrophages in the splenic red pulp and there appeared to be more CD11c positive cells in the red pulp than α_d positive cells. The majority of macrophages expressing α_d also expressed CD11c even though a small subset of α_d positive macrophages were observed that did not express CD11c. There was also a population of CD11c positive macrophages that did not express α_d.

Immunohistology analysis therefore indicates that there appears to be an upregulation of CD11c expression in the spleen of phenylhydrazine treated animals compared to the saline controls on day 4. Expression of the other integrins, CD11a,

CD11b and α_d, however, appears to be unaffected by the phenylhydrazine treatment. Treatment with polyclonal "I" domain α_d antibody also showed no effect on the uptake of rbcs by the red pulp macrophages, but the majority of macrophages that are engulfing rbcs are α_d positive. The α_d positive macrophages which had engulfed damaged rbcs were not present in spleens collected 7 days after phenylhydrazine administration.

A double label experiment was then performed on the day 4 phenylhydrazine-treated rat spleens using an apoptosis assay and ICC with biotin-conjugated antibody 205C. Tissue from normal rat and day four phenylhydrazine-treated rats were sectioned at 4 microns thickness and air dried on Superfrost Plus slides (VWR Scientific) at room temperature for 15 minutes and stored at -20°C. Prior to use, slides were warmed to 50°C. Warmed slides were placed in buffer containing 100 ml 1X TBS, 1.1 ml 30% H_2O_2 (Sigma), 1 ml 10% NaN_3 (Sigma) for 15 minutes at room temperature to remove endogenous peroxidase activity. Each section was blocked using 150 µl of a solution containing 20% normal rat serum (Harlan Bioproducts), 2% BSA (Sigma) in 1x TBS for 10 minutes at 37°C, after which the solution was gently blotted from the sections. Each section was incubated for 30 minutes at 37 °C with 75 µl biotinylated hamster anti-rat α_d antibody 205C at a protein concentration of 26.6 µg/ml diluted in blocking solution. Sections were then washed three times for five minutes each in 1X TBS to remove unbound antibody. Excess TBS was removed by aspirating around the tissue following the final wash. Alkaline phosphatase-conjugated avidin/biotin complex (Vector Laboratories) prepared according to the manufacturer's instructions was applied to each section for 20 minutes at 37°C. After incubation, slides were washed two times for five minutes each in 1X TBS. Sections were fixed for five minutes with 4% paraformaldehyde (Sigma) at 4°C. Sections were then rinsed in 1X PBS and placed in CSK buffer (100 mM NaCl, 300 mM sucrose, 10 mM pipes pH 6.8, 3 mM $MgCl_2$, 0.5% Triton-X-100®) for two minutes at 4°C. Sections were rinsed in 1X PBS for two minutes at room temperature after which the sections were washed three times for five minutes each with 1X PBS. TUNEL reaction mixture (Boehringer Mannheim) was applied to each section for 60 minutes at 37°C. After incubation, the sections were washed three times in 1X PBS for 5 minutes each wash. The apoptosis kit methodology is similar to *in situ* hybridization; the TUNEL reagent (which is FITC conjugated) hybridizes to

"nicked" DNA. Converter-POD (a peroxidase conjugated antibody which recognizes the FITC tag on the TUNEL reagent) was applied to each section for 30 minutes at 37 °C and the sections were washed three times for five minutes each with 1X PBS. AEC (Vector Laboratories) was applied and color development stopped by immersion in water. Sections were mounted with Aquamount (VWR).

In the model, numerous cells in both the red and white pulp regions of the spleen were undergoing apoptosis, but large macrophages in the red pulp (which expressed α_d and disappeared on day 7 of the model) that had engulfed RBCs were not found to be undergoing apoptosis.

Cell Type Analysis of Rat α_d Expression on Normal Rat Spleen

In order to determine which rat cell types express α_d, a double label staining was performed on normal rat spleen. Sections of normal rat spleen were prepared as described above through the rat serum blocking step. After the addition of primary cell marker antibodies, alkaline phosphatase-conjugated goat anti-mouse antibody (Jackson Laboratories) was diluted 1:500, in the same diluent used for the primary antibodies, and 75 µl was applied to each section for 30 minutes at room temperature. Slides were washed two times in 1X TBS for five minutes each wash. Alkaline phosphatase conjugated donkey anti-goat antibody (Jackson Laboratories) was diluted 1:300, in antibody diluent, and 75 µl was applied to each section for 30 minutes at room temperature. After washing and blocking as above, each section received 75 µl of biotinylated hamster anti-rat α_d antibody (205C), at a protein concentration of 20 µg/ml, for 1 hour 45 minutes each and then washed to remove unbound antibody. Excess TBS was removed by aspirating around the tissue following the final wash. Peroxidase conjugated goat anti-biotin (Vector Laboratories) was diluted 1:200, in antibody diluent, and 75 µl was applied to each section for 45 minutes at room temperature. Slides were washed two times 1X TBS for five minutes each wash. AEC substrate (Vector Laboratories) was applied and color development was stopped by immersion in water. Fast Blue substrate (Vector Laboratories) was then applied and color development was stopped by immersion in water. Slides were then mounted with Aquamount (Baxter).

Dual antibody immunocytochemistry was performed using antibodies to

CD5, CD2, CD4, CD8, NK marker, or HIS 45 (a T cell marker) in conjunction with anti-α_d antibody in an attempt to determine the phenotype of cells expressing α_d. No double labeled cells were detected with CD2/α_d or HIS 45/α_d. In the splenic red pulp of a normal rat α_d labeled clusters of small cells which were found to also express CD5. It was not determined if the CD5 positive cells were T cells or B cells. A small population of α_d expressing cells in the red pulp were also determined to express CD4. In addition, a subset of NK cells and CD8 positive cells were also identified that expressed α_d. Therefore, α_d expression in the spleen was found on a subset of T cells, possibly a subset of B cells, a subset of NK cells, and subset of macrophages.

Expression of α_d on Large Granulocytic Leukocytes
(LGL) Tumor Cells from the F344 Rat Model

A rat model for LGL-leukemia was designed in the F344 rat using tumor cells received from the National Cancer Institute which were injected intravenously into 3 male F344 rats (1 million cells each). The disease took three months to manifest, at which time several of the animals were sacrificed and tissues examined by FACS and histochemical analysis.

For FACS analysis, a portion of the spleen was removed and a single-cell suspension prepared as described in example below. Briefly, the splenic tissue was minced into smaller pieces with scissors and passed through a wire mesh screen in the presence of D-PBS. The cells were pelleted by centrifugation and resuspended in 30 ml D-PBS. Histopaque gradients (Sigma) were prepared by layering 5.0 ml of the cell suspension over 5.0 ml of Histopaque within a 15 ml centrifuge tube. The gradients were centrifuged for 30 minutes at 1500 rpm using a Beckman Tabletop Centrifuge and the cellular layer collected, washed once in D-PBS, and counted by hemacytometer. The cells were resuspended in FACS buffer (RPMI-1640/2% FBS, 0.2% sodium azide) to a density of 1×10^6 cells/sample.

The cells were two-color stained by incubation with the hamster anti-rat α_d antibody 205C conjugated to biotin (10 µg/ml) and one of a series of antibodies against rat cellular markers that were FITC conjugated. These second antibodies included anti-macrophage-FITC, anti-CD3-FITC, and anti-IgM (B-cell)-FITC antibodies (all from

PharMingen), in addition to a FITC-conjugated antibody with NK cell specificity (Harlan). The FITC conjugated antibodies were each used at 10 µl/sample.

The samples were first incubated on ice for 30 minutes with 205C-biotin antibody, washed three times in FACS buffer, and resuspended in 1.0 ml FACS buffer. The FITC conjugates were added along with 5 µl streptavidin-PE (Pharmigen) and the samples placed on ice for 30 minutes. After incubation, the samples were washed three times in FACS buffer and resuspended in 200 µl FACS buffer. Samples were examined using a Becton Dickinson FACscan and the data analyzed using Lysis II software (Becton Dickinson).

The results overwhelmingly demonstrated expression of α_d on the surface of NK, or LGL, cells. Cells which stained positive for B-cell and T-cell markers did not reveal α_d expression and cells which stained using the macrophage marker showed only a slight degree of α_d expression. It is believed that, at this point in the disease, the spleen is composed predominantly of NK tumor cells, consistent with the observation that a large population of spleen cells stained for expression of both the NK marker and α_d.

These observations were also consistent with results from a parallel experiment using peripheral blood cells also collected from the same animals and processed as above for FACS. Results using peripheral blood cells indicated that circulating NK cells also express α_d, while cells expressing other cellular markers in the blood did not show α_d expression. Results using peripheral blood cells, however, were not as dramatic as the splenic cell results presumably due to a difference in the percentage of different cell types present in the spleen and peripheral blood.

In subsequent FACS analysis of rat spleen cells from these model animals, identical results were obtained. Upon further analysis using the above method of cell preparation, the LGL tumor cells have also shown staining for expression of CD18 as well as CD11a, CD11b, and CD11c.

Histochemical analysis was carried out on both normal and NK F344 diseased tissue using the ICC procedure described above. Preliminary data indicated that α_d was expressed in NK F344 tumor lung and liver tissue, but was either not detected or was expressed in very low levels in normal respective tissue. Diseased lung tissue showed expression of α_d on small and large clusters of cells, as well as individual cells throughout

the lung. The NK F344 liver showed weak labeling around vessels and other cells throughout the tissue. Antibodies to the other β_2 integrins indicated these molecules are expressed at similar levels in both normal and diseased tissue although labeling patterns did vary.

In parallel analyses, the normal rat thymus showed slight α_d expression in scattered cells in the cortex, while the F344 NK thymus showed an increased level of α_d expression. While the normal spleen showed expression in the red pulp, the NK spleen had cluster labeling throughout the tissue.

The NK spleen was then tested at weekly intervals from onset of the disease which indicated that the level of expression of α_d increased up until the third week and then dropped off at the fourth week.

Example 27
Assay for Inhibition of NK-Tumor Cell-Induced Target Cell Lysis Using Anti-α_d Monoclonal Antibodies

A specific function of NK cells is to target and kill virally-infected and foreign cells. To assay the ability of NK cells to lyse a specific target cell, target cells are labeled with ^{51}chromium and as lysis occurs, increasing radioactivity is detected in the medium. It was postulated that α_d, previously shown to be expressed on NK cells, might participate in NK targeted cell killing. To test this hypothesis, tumor cells were pre-incubated with α_d antibodies in order to assess the role of α_d in a functional assay.

Preparation of α_d Positive NK-tumor Effector Cells

F344 rats were injected with NK tumor cells, originally obtained from the National Cancer Institute and passaged through animals three to four weeks prior to removal of the spleen. The spleen was removed and was minced into small pieces which were passed through a wire-mesh screen in the presence of D-PBS. The resultant cell suspension was centrifuged at 1500 rpm in a Beckman tabletop centrifuge for 10 minutes at room temperature. The supernatant was aspirated and the cell pellet resuspended in 30 ml D-PBS.

Histopaque separation of mononuclear cells from blood was then carried out as follows. Five ml Histopaque (Sigma) was added to six 15 ml centrifuge tubes on

top of which was layered 5.0 ml of the cell suspension described above. The cells were centrifuged at 1500 rpm for 30 minutes in a Beckman Tabletop centrifuge at room temperature. The cellular layer was collected, pooled and counted by hemacytometer. Several dilutions of the isolated tumor cells were prepared in D-PBS buffer and subsequently incubated in the presence or absence of anti-rat α_d antibodies at a concentration of 50 µg/ml. Control antibodies included an anti-rat CD18 antibody and an anti-rat ICAM-1 antibody which were also incubated with the cells at 50 µg/ml concentration. Tumor cells were pre-incubated with antibodies at 37°C for approximately 30 minutes prior to the assay.

Chromium Labeling of Yak-1 Target Cells

Yak-1 cells (ATCC), a mouse lymphoma cell line, were cultured in 10% FBS/RPMI 1640. Cells were harvested by centrifugation and resuspended at a density of approximately 1 x 10^7 cells in 1.5 to 4.0 ml of RPMI "test media" made from 500 ml RPMI 1640, 5 ml Pen-Strep antibiotic solution, and 10 ml FBS. Approximately 200 to 300 µCi of ^{51}chromium was added to the Yak-1 cell suspension which were then incubated at 37°C for 45 to 60 minutes with gentle mixing. Following incubation, the volume was increased to 50 ml with test media and the cells pelleted by centrifugation. The supernatant was discarded and cells were suspended in 1 to 3 ml test media and adjusted to a density of 5 x 10^4 cells/ml. Non-labeled Yak-1 cells were also prepared at a concentration of 1 x 10^7 cell/ml to use as autologous controls.

The activity of labeled cells was determined by assaying 100 µl of the labeled cell suspension in triplicate using a gamma counter.

Short-Term Chromium Release Assay

NK effector cells of each dilution were plated in triplicate in a volume of 100 µl test media in a 96-well microtiter plate and 100 µl labeled Yak-1 cells were added to each well. Autologous, *i.e.*, spontaneous or background, release was obtained by incubating 100 µl of labeled cells with non-labeled Yak cells at each of the effector dilutions. Total ^{51}chromium release was obtained by adding 1.0% Triton® to target cells in one set of wells. Spontaneous release was measured by the amount of ^{51}chromium

found in wells with only target cells. Incubation of effector/target cells was carried out for four hours at 37°C after which the plates were centrifuged and 100 μl supernatant collected from each well and radioactivity measured.

Cytolytic activity was calculated by using the following formula:

$$\%\ \text{cytolytic activity} = \frac{\text{cmp of sample - spontaneous release cpm}}{\text{100\% release cmp - spontaneous release cmp}} \times 100$$

Results indicated that neither hamster anti-rat α_d antibodies (including 199M and 205C) nor mouse anti-rat α_d antibodies (226A, 226B, 226C, 226D, 226F, 226G, 226H, and 226I) effected the ability of NK tumor cells to kill or lyse the labeled target cells.

Example 28
Isolation of Mouse cDNA Clones

Isolation of a mouse α_d homolog was attempted.

Cross-species hybridization was performed using two PCR-generated probes: a 1.5 kb fragment corresponding to bases 522 to 2047 from human clone 19A2 (SEQ ID NO: 1), and a 1.0 kb rat fragment which corresponds to bases 1900 to 2900 in human clone 19A2 (SEQ ID NO: 1). The human probe was generated by PCR using primer pairs designated ATM-2 and 9-10.1 set out in SEQ ID NOS: 38 and 39, respectively; the rat probe was generated using primer pairs 434L and 434R, set out in SEQ ID NOS: 34 and 35, respectively. Samples were incubated at 94°C for 4 minutes and subjected to 30 cycles of the temperature step sequence: 94°C; 50°C 2 minutes; 72°C, 4 minutes.

5´-GTCCAAGCTGTCATGGGCCAG-3´ (SEQ ID NO: 38)
5´-GTCCAGCAGACTGAAGAGCACGG-3´ (SEQ ID NO: 39)

The PCR products were purified using the Qiagen Quick Spin kit according to manufacturer's suggested protocol, and approximately 180 in DNA was

labeled with 200 μCi [^{32}P]-dCTP using a Boehringer Mannheim Random Primer Labeling kit according to manufacturer's suggested protocol. Unincorporated isotope was removed using a Centri-sep Spin Column (Princeton Separations, Adelphia, NJ) according to manufacturer's suggested protocol. The probes were denatured with 0.2 N NaOH and neutralized with 0.4 M Tris-HCl, pH 8.0, before use.

A mouse thymic oligo dT-primed cDNA library in lambda ZAP® II (Stratagene) was plated at approximately 30,000 plaques per 15 cm plate. Plaque lifts on nitrocellulose filters (Schleicher & Schuell, Keene, NH) were incubated at 50°C with agitation for 1 hour in a prehybridization solution (8 ml/lift) containing 30% formamide. Labeled human and rat probes were added to the prehybridization solution and incubation continued overnight at 50°C. Filters were washed twice in 2X SSC/0.1% at room temperature, once in 2X SSC/0.1% SDS at 37°C, and once in 2X SSC/0.1% SDS at 42°C. Filters were exposed on Kodak X-Omat AR film at -80°C for 27 hours with an intensifying screen.

Four plaques giving positive signals on duplicate lifts were restreaked on LB medium with magnesium (LBM)/carbenicillin (100 mg/ml) plates and incubated overnight at 37°C. The phage plaques were lifted with Hybond® filters (Amersham), probed as in the initial screen, and exposed on Kodak X-Omat AR film for 24 hours at -80°C with an intensifying screen.

Twelve plaques giving positive signals were transferred into low Mg^{++} phage diluent containing 10 mM Tris-HCl and 1 mM $MgCl_2$. Insert size was determined by PCR amplification using T3 and T7 primers (SEQ ID NOS: 13 and 14, respectively) and the following reaction conditions. Samples were incubated at 94°C for 4 minutes and subjected to 30 cycles of the temperature step sequence: 94°C, for 15 seconds; 50°C, for 30 seconds; and 72°C for 1 minute.

Six samples produced distinct bands that ranged in size from 300 bases to 1 kb. Phagemids were released via co-infection with helper phage and recircularized to generate Bluescript® SK$^-$ (Stratagene). The resulting colonies were cultured in LBM/carbenicillin (100 mg/ml) overnight. DNA was isolated with a Promega Wizard® miniprep kit (Madison, WI) according to manufacturer's suggested protocol. *Eco*RI restriction analysis of purified DNA confirmed the molecular weights which were

detected using PCR. Insert DNA was sequenced with M13 and M13 reverse.1 primers set out in SEQ ID NOS: 40 and 41, respectively.

5´-TGTAAAACGACGGCCAGT-3´ (SEQ ID NO: 40)

5´-GGAAACAGCTATGACCATG-3´ (SEQ ID NO: 41)

Sequencing was performed as described in Example 4.

Of the six clones, only two, designated 10.3-1 and 10.5-2, provided sequence information and were identical 600 bp fragments. The 600 bp sequence was 68% identical to a corresponding region of human α_d, 40% identical to human CD11a, 58% identical to human CD11c, and 54% identical to mouse CD11b. This 600 bp fragment was then utilized to isolate a more complete cDNA encoding a putative mouse α_d homolog.

A mouse splenic cDNA library (oligo dT and random-primed) in lambda Zap® II (Stratagene) was plated at 2.5 x 10^4 phage/15 cm LBM plate. Plaques were lifted on Hybond® nylon transfer membranes (Amersham), denatured with 0.5 M NaOH/1.5 M NaCl, neutralized with 0.5 M Tris Base/1.5 M NaCl/11.6 HCl, and washed in 2X SSC. The DNA was cross-linked to filters by ultraviolet irradiation.

Approximately 500,000 plaques were screened using probes 10.3-1 and 10.5-2 previously labeled as described *supra*. Probes were added to a prehybridization solution and incubated overnight at 50°C. The filters were washed twice in 2X SSC/0.1% SDS at room temperature, once in 2X SSC/0.1% SDS at 37°C, and once in 2X SSC/0.1% SDS at 42°C. Filters were exposed on Kodak X-Omat AR film for 24 hours at -80°C with an intensifying screen. Fourteen plaques giving positive signals on duplicate lifts were subjected to a secondary screen identical to that for the initial screen except for additional final high stringency washes in 2X SSC/ 0.1% SDS at 50°C, in 0.5X SSC/0.1% SDS at 50°C, and at 55°C in 0.2X SSC/0.1% SDS. The filters were exposed on Kodak X-Omat AR film at -80°C for 13 hours with an intensifying screen.

Eighteen positive plaques were transferred into low Mg^{++} phage diluent and insert size determined by PCR amplification as described above. Seven of the

samples gave single bands that ranged in size from 600 bp to 4 kb. *Eco*RI restriction analysis of purified DNA confirmed the sizes observed from PCR and the DNA was sequenced with primers M13 and M13 reverse.1 (SEQ ID NOS: 40 and 41, respectively).

One clone designated B3800 contained a 4 kb insert which corresponded to a region 200 bases downstream of the 5´ end of the human α_d 19A2 clone and includes 553 bases of a 3´ untranslated region. Clone B3800 showed 77% identity to a corresponding region of human α_d, 44% identity to a corresponding region of human CD11a, 59% identity to a corresponding region of human CD11c, and 51% identity to a corresponding region of mouse CD11b. The second clone A1160 was a 1.2 kb insert which aligned to the 5´ end of the coding region of human α_d approximately 12 nucleic acids downstream of the initiating methionine. Clone A1160 showed 75% identity to a corresponding region of human α_d, 46% identity to a corresponding region of human CD11a, 62% identity to a corresponding region of human CD11c, and 66% identity to a corresponding region of mouse CD11b.

Clone A1160, the fragment closer to the 5´ end of human clone 19A2, is 1160 bases in length, and shares a region of overlap with clone B3800 starting at base 205 and continuing to base 1134. Clone A1160 has a 110-base insertion (bases 704-814 of clone A1160) not present in the overlapping region of clone B3800. This insertion occurs at a probable exon-intron boundary [Fleming, *et al.*, *J.Immunol. 150*:480-490 (1993)] and was removed before subsequent ligation of clones A1160 and B3800.

Rapid Amplification of 5´ cDNA End of the Putative Mouse α_d Clone

RACE PCR [Frohman, "RACE: Rapid Amplification of cDNA Ends," in PCR Protocols: A Guide to Methods and Applications, Innis, *et al.* (eds.) pp. 28-38, Academic Press:New York (1990)] was used to obtain missing 5´ sequences of the putative mouse α_d clone, including 5´ untranslated sequence and initiating methionine. A mouse splenic RACE-Ready kit (Clontech, Palo Alto, CA) was used according to the manufacturer's suggested protocol. Two antisense, gene-specific primers, A1160 RACE1-primary and A1160 RACE2-nested (SEQ ID NOS: 42 and 43), were designed

to perform primary and nested PCR.

5′-GGACATGTTCACTGCCTCTAGG-3′ (SEQ ID NO: 42)

5′-GGCGGACAGTCAGACGACTGTCCTG-3′ (SEQ ID NO: 43)

The primers, SEQ ID NOS: 42 and 43, correspond to regions starting 302 and 247 bases from the 5′ end, respectively. PCR was performed as described, *supra*, using the 5′ anchor primer (SEQ ID NO: 44) and mouse spleen cDNA supplied with the kit.

5′-CTGGTTCGGCCCACCTCTGAAGGTTCCAGAATCGATAG-3′ (SEQ ID NO: 44)

Electrophoresis of the PCR product revealed a band approximately 280 bases in size, which was subcloned using a TA cloning kit (Invitrogen) according to manufacturer's suggested protocol. Ten resulting colonies were cultured, and the DNA isolated and sequenced. An additional 60 bases of 5′ sequence were identified by this method, which correspond to bases 1 to 60 in SEQ ID NO: 45.

Characteristics of the Mouse cDNA and Predicted Amino Acid Sequence

A composite sequence of the mouse cDNA encoding a putative homolog of human α_d is set out in SEQ ID NO: 45. Although homology between the external domains of the human and mouse clones is high, homology between the cytoplasmic domains is only 30%. The observed variation may indicate C-terminal functional differences between the human and mouse proteins. Alternatively, the variation in the cytoplasmic domains may result from splice variation, or may indicate the existence of an additional β_2 integrin gene(s).

At the amino acid level, the mouse cDNA predicts a protein (SEQ ID NO: 46) with 28% identity to mouse CD11a, 53% identity to mouse CD11b, 28% identity to human CD11a, 55% identity to human CD11b, 59% identity to human

CD11c, and 70% identity to human α_d. Comparison of the amino acid sequences of the cytoplasmic domains of human α_d and the putative mouse homolog indicates regions of the same length, but having divergent primary structure. Similar sequence length in these regions suggests species variation rather than splice variant forms. When compared to the predicted rat polypeptide, Example 20, *supra*, mouse and rat cytoplasmic domains show greater than 60% identity.

Example 29
Isolation of Additional Mouse αd cDNA Clones for Sequence Verification

In order to verify the nucleic and amino acids sequences describe in Example 28 for mouse α_d, additional mouse sequences were isolated for the purposes of confirmation.

Isolation of mouse cDNA by hybridization with two homologous α_d probes (3´ and 5´) was performed using both a mouse splenic random primed library and an oligo dT-primed cDNA library in lambda ZAP® II (Strategene). The library was plated at 5 x 10^5 phage per 15 cm LBM plate. Plaques were lifted on Hybond® nylon membranes (Amersham), and the membranes were denatured (0.5 M NaOH/1.5 M NaCl), neutralized (0.5 M Tris Base/1.5 M NaCl / 11.6 M HCl) and washed (2X SSC salt solution). DNA was cross-lined to filters by ultraviolet irradiation.

Probes were generated using primers described below in a PCR reaction under the following conditions. Samples were held at 94°C for 4 minutes and then run through 30 cycles of the temperature step sequence (94°C for 15 seconds; 50°C for 30 seconds; 72°C for 1 minute in a Perkin-Elmer 9600 thermocycler).

The 3´ probe was approximately 900 bases long and spanned a region from nucleotides 2752 to 3651 (in SEQ ID NO: 1) (5´ → 3´) and was produced with primers 11.b-1/2FOR11 and 11.b-1/2REV2 as shown in SEQ ID NOS: 69 and 74, respectively. This probe was used in a first set of lifts.

The 5´ probe was approximately 800 bases long and spanned a region from nucleotides 149 to 946 (in SEQ ID NO: 1) (5´ → 3´) and was produced with primers 11.b-1/2FOR1 and 11.a-1/1REV1 as shown in SEQ ID NOS: 50 and 85, respectively). This probe was used in a second set of lifts.

In a third set of lifts, both probes described above were used together on the same plates.

Approximately 500,000 plaques were screened using the two probes from above which were labeled in the same way as described in Example 20. Labeled probes were added to a prehybridization solution, containing 45% formamide, and incubated overnight at 50°C. Filters were washed twice in 2X SSC/0.1% SDS at room temperature (22°C). A final wash was carried out in 2X SSC/0.1% SDS at 50°C. Autoradiography was for 19 hours at -80°C on Kodak X-Omat AR film with an intensifying screen.

Thirteen plaques giving positive signals on at least duplicate lifts were subjected to a secondary screen performed as described for the initial screen except that both the 3´ and 5´ labeled probes were used for hybridization and an additional final wash was incorporated using 2X SSC/0.1% SDS at 65´C. Autoradiography was performed as described above for 2.5 hours.

Thirteen plaques (designated MS2P1 through MS2P13) giving positive signals were transferred into low Mg^{++} phage diluent. Insert size was determined by PCR amplification (Perkin-Elmer 9600 thermocycler) using T3 and T7 primers which anneal to Bluescript® phagemid in ZAP® II (sequence previously described) under the same conditions shown above. Band sizes ranged from 500 bases to 4Kb. Phagemids were isolated, prepared, and sequenced with M13 and M13 reverse.1 primers (SEQ ID NOS: 40 and 41, respectively). Five of the thirteen clones; MS2P-3, MS2P-6, MS2P-9, MS2P-12, and MS2P-13, were sequenced, and together, represented a region from approximately base 200 at the 5´ end to about 300 bases past a first stop codon at the 3´ end.

Automated sequencing was performed as described in Example 4 by first using M13 and M13 reverse.1 primers (SEQ ID NOS: 40 and 41, respectively) to sequence the ends of each clone and to determine its position relative to construct #17 (SEQ ID NO: 45). Each clone was then completely sequenced using the appropriate primers (listed below) for that particular region.

11.b-1/2FOR1 5´-GCAGCCAGCTTCGGACAGAC-3´ (SEQ ID NO: 50)

11.a-1/1FOR2	5´-CCGCCTGCCACTGGCGTGTGC-3´	(SEQ ID NO: 60)
11.a-1/1FOR3	5´-CCCAGATGAAGGACTTCGTCAA-3´	(SEQ ID NO: 61)
11.b-1/2FOR4	5´-GCTGGGATCATTCGCTATGC-3´	(SEQ ID NO: 62)
11.b-1/2FOR5	5´-CAATGGATGGACCAGTTCTGG-3´	(SEQ ID NO: 63)
11.b-1/2FOR6	5´-CAGATCGGCTCCTACTTTGG-3´	(SEQ ID NO: 64)
11.b-1/2FOR7	5´-CATGGAGCCTCGAGACAGG-3´	(SEQ ID NO: 65)
11.b-1/2FOR8	5´-CCACTGTCCTCGAAGCTGGAG-3´	(SEQ ID NO: 66)
11.b-1/2FOR9	5´-CTTCGTCCTGTGCTGGCTGTGGGCTC-3	(SEQ ID NO: 67)
11.b-1/2FOR10	5´-CGCCTGGCATGTGAGGCTGAG-3´	(SEQ ID NO: 68)
11.b-1/2FOR11	5´-CCGTGATCAGTAGGCAGGAAG-3´	(SEQ ID NO: 69)
11.b-1/2FOR12	5 -GTCACAGAGGGAACCTCC-3´	(SEQ ID NO: 70)
11.b-1/2FOR13	5´-GCTCCTGAGTGAGGCTGAAATCA-3´	(SEQ ID NO: 71)
11.b-1/2FOR14	5´-GAGATGCTGGATCTACCATCTGC-3´	(SEQ ID NO: 72)
11.b-1/2FOR15	5´-CTGAGCTGGGAGATTTTTATGG-3´	(SEQ ID NO: 73)
11.b-1/2REV2	5´-GTGGATCAGCACTGAAATCTG-3´	(SEQ ID NO: 74)
11.b-1/2REV3	5´-CGTTTGAAGAAGCCAAGCTTG-3´	(SEQ ID NO: 75)
11.b-1/2REV4	5´-CACAGCGGAGGTGCAGGCAG-3´	(SEQ ID NO: 76)
11.b-1/2REV5	5´-CTCACTGCTTGCGCTGGC-3´	(SEQ ID NO: 77)
11.b-1/2REV6	5´-CGGTAAGATAGCTCTGCTGG-3´	(SEQ ID NO: 78)
11.b-1/2REV7	5´-GAGCCCACAGCCAGCACAGG-3´	(SEQ ID NO: 79)
11.b-1/2REV8	5´-GATCCAACGCCAGATCATACC-3´	(SEQ ID NO: 80)
11.b-1/2REV9	5´-CACGGCCAGGTCCACCAGGC-3´	(SEQ ID NO: 81)
11.b-1/2REV10	5´-CACGTCCCCTAGCACTGTCAG-3´	(SEQ ID NO: 82)
11.b-1/2REV11	5´-CCATGTCCACAGAACAGAGAG-3´	(SEQ ID NO: 51)
11.b-1/2REV12	5´-TTGACGAAGTCCTTCATCTGGG-3´	(SEQ ID NO: 83)
11.b-1/2REV13	5´-GAACTGCAAGCTGGAGCCCAG-3´	(SEQ ID NO: 84)
11.a-1/1REV1	5´-CTGGATGCTGCGAAGTGCTAC-3´	(SEQ ID NO: 85)
11.a-1/1REV2	5´-GCCTTGGAGCTGGACGATGGC-3´	(SEQ ID NO: 86)

Sequences were edited, aligned, and compared to a previously isolated mouse α_d

sequence (construct #17, SEQ ID NO: 45).

Alignment of the new sequences revealed an 18 base deletion in construct #17 beginning at nucleotide 2308; the deletion did not cause a shift in the reading frame. Clone MS2P-9, sequenced as described above, also revealed the same 18 base deletion. The deletion has been observed to occur in 50% of mouse clones that include the region but has not been detected in rat or human α_d clones. The eighteen base deletion is characterized by a 12 base palindromic sequence AAGCAGGAGCTCCTGTGT (SEQ ID NO: 91). This inverted repeat in the nucleic acid sequence is self-complementary and may form a loop out, causing cleavage during reverse transcription. The mouse α_d sequence which includes the additional 18 bases is set forth in SEQ ID NO: 52; the deduced amino acid sequence is set forth in SEQ ID NO: 53.

Example 30

***In situ* Hybridizations in Mouse**

Tissue distribution was then determined for mouse α_d in order to provide a comparison to that in humans, described in Example 6.

A single stranded 200 bp mRNA probe was generated from a DNA template, corresponding to nucleotides 3460 to 3707 in the cytoplasmic tail region of the murine cDNA, by *in vitro* RNA transcription incorporating ^{35}S-UTP (Amersham).

Whole mouse embryos (harvested at days 11-18 after fertilization) and various mouse tissues, including spleen, kidney, liver, intestine, and thymus, were hybridized *in situ* with the radiolabeled single-stranded mRNA probe.

Tissues were sectioned at 6 μm thickness, adhered to Vectabond (Vector Laboratories, Inc., Burlingame, CA) coated slides, and stored at -70°C. Prior to use, slides were removed from -70°C and placed at 50°C for approximately 5 minutes. Sections were fixed in 4% paraformaldehyde for 20 minutes at 4°C, dehydrated with an increasing ethanol gradient (70-95-100%) for 1 minute at 4°C at each concentration, and air dried for 30 minutes at room temperature. Sections were denatured for 2 minutes at 70°C in 70% formamide/2X SSC, rinsed twice in 2X SSC, dehydrated with the ethanol gradient described *supra* and air dried for 30 minutes. Hybridization was

carried out overnight (12-16 hours) at 55°C in a solution containing ^{35}S-labeled riboprobes at 6 x 10^5 cpm/section and diethylpyrocarbonate (DEPC)-treated water to give a final concentration of 50% formamide, 0.3 M NaCl, 20 mM Tris-HCl, pH 7.5, 10% dextran sulfate, 1X Denhardt's solution, 100 mM dithiothreitol (DTT) and 5 mM EDTA. After hybridization, sections were washed for 1 hour at room temperature in 4X SSC/10 mM DTT, 40 minutes at 60°C in 50% formamide/2X SSC/10 mM DTT, 30 minutes at room temperature in 2X SSC, and 30 minutes at room temperature in 0.1X SSC. The sections were dehydrated, air dried for 2 hours, coated with Kodak NTB2 photographic emulsion, air dried for 2 hours, developed (after storage at 4°C in complete darkness) and counterstained with hematoxylin/eosin.

Spleen tissue showed a strong signal primarily in the red pulp. This pattern is consistent with that of tissue macrophage distribution in the spleen, but does not exclude other cell types.

Example 31
Generation of Mouse Expression Constructs

In order to construct an expression plasmid including mouse cDNA sequences exhibiting homology to human α_d, inserts from clones A1160 and B3800 were ligated. Prior to this ligation, however, a 5´ leader sequence, including an initiating methionine, was added to clone A1160. A primer designated "5´ PCR leader" (SEQ ID NO: 47) was designed to contain: (1) identical nonspecific bases at positions 1-6 allowing for digestion; (2) a *Bam*HI site (underlined in SEQ ID NO: 47) from positions 7-12 to facilitate subcloning into an expression vector; (3) a consensus Kozak sequence from positions 13-18, (4) a signal sequence including a codon for an initiating methionine (bold in SEQ ID NO: 47), and (5) an additional 31 bases of specifically overlapping 5´ sequence from clone A1160 to allow primer annealing. A second primer designated "3´ end frag" (SEQ ID NO: 48) was used with primer "5´ PCR leader" to amplify the insert from clone A1160.

5´-AGTTACGGATCCGGCACCATGAC-

-CTTCGGCACTGTGATCCTCCTGTGTG-3´ (SEQ ID NO: 47)

5´-GCTGGACGATGGCATCCAC-3´ (SEQ ID NO: 48)

The resulting PCR product did not digest with *Bam*HI, suggesting that an insufficient number of bases preceded the restriction site, prohibiting recognition by the enzyme. The length of the "tail" sequence preceding the *Bam*HI site in the 5´ primer (SEQ ID NO: 47) was increased and PCR was repeated on the amplification product from the first PCR. A 5´ primer, designated mAD.5´.2 (SEQ ID NO: 49), was designed with additional nonspecific bases at positions 1-4 and an additional 20 bases specifically overlapping the previously employed "5´ PCR leader" primer sequences.

5´-GTAGAGTTACGGATCCGGCACCAT-3´ (SEQ ID NO: 49)

Primers "mAD.5´.2" and "3´ end frag" were used together in PCR with the product from the first amplification as template. A resulting secondary PCR product was subcloned into plasmid pCRtmII (Invitrogen) according to manufacturer's suggested protocol and transformed into competent One shot cells (Invitrogen). One clone containing the PCR product was identified by restriction enzyme analysis using *Bam*HI and *Eco*RI and sequenced. After the sequence was verified, the insert was isolated by digestion with *Bam*HI and *Eco*RI and gel purified.

The insert from clone B3800 was isolated by digestion with *Eco*RI and *Not*I, gel purified, and added to a ligation reaction which included the augmented A1160 *Bam*HI/*Eco*RI fragment. Ligation was allowed to proceed for 14 hours at 14°C. Vector pcDNA.3 (Invitrogen), digested with *Bam*HI and *Not*I, was added to the ligation reaction with additional ligase and the reaction was continued for another 12 hours. An aliquot of the reaction mixture was transformed into competent *E. coli* cells, the resulting colonies cultured, and one positive clone identified by PCR analysis with the primers 11.b-1/2FOR1 and 11.b-1/2REV11 (SEQ ID NOS: 50 and 51, respectively). These primers bridge the A1160 and B3800 fragments, therefore

detection of an amplification product indicates the two fragments were ligated. The sequence of the positive clone was verified with the primers set out in SEQ ID NOS: 50 and 51, which amplify from base 100 to 1405 after the initiating methionine.

Example 32
Construction of a Knock-out Mouse

In order to more accurately assess the immunological role of the protein encoded by the putative mouse α_d cDNA, a "knock-out" mouse is designed wherein the genomic DNA sequence encoding the putative α_d homolog is disrupted by homologous recombination. The significance of the protein encoded by the disrupted gene is thereby assessed by the absence of the encoded protein. Generation of "knock-out" mice is described in Deng, *et al.*, *Mol.Cell.Biol.* *13*:2134-2140 (1993).

Design of such a mouse begins with construction of a plasmid containing sequences to be "knocked out" by homologous recombination events. A 750 base pair fragment of the mouse cDNA (corresponding to nucleotides 1985 to 2733 in SEQ ID NO: 45) was used to identify a mouse genomic sequence encoding the putative mouse α_d homolog from a λFIXII genomic library. Primary screening resulted in 14 positive plaques, seven of which were confirmed by secondary screening. Liquid lysates were obtained from two of the plaques giving the strongest signal and the λ DNA was isolated by conventional methods. Restriction mapping and Southern analysis confirmed the authenticity of one clone, designated 14-1, and the insert DNA was isolated by digestion with *Not*I. This fragment was cloned into Bluescript® SKII$^+$.

In order to identify a restriction fragment of approximately 9 to 14 kb, a length reported to optimize the probability of homologous recombination events, Southern hybridization was performed with the 750 bp cDNA probe. Prior to hybridization, a restriction map was constructed for clone 14-1. A 12 kb fragment was identified as a possible candidate and this fragment was subcloned into pBluescript® SKII$^+$ in a position wherein the mouse DNA is flanked by thymidine kinase encoding cassettes. Further analysis of this clone with an I domain probe (corresponding to nucleotides 454-1064 in SEQ ID NO: 45) indicated that the clone did not contain I domain encoding sequences.

Using the same I domain probe, the λFIXII genomic library was rescreened. Initially, six positive clones were detected, one of which remained positive upon secondary screening. DNA isolated from this clone reacted strongly in Southern analysis with an I domain probe. No reactivity was detected using the original 750 bp probe, however, indicating that this clone included regions 5´ to nucleotides 1985-2773 of SEQ ID NO: 45.

Alternatively, the lack of hybridization to the 750 bp probe may have suggested that the clone was another member of the integrin family of proteins. To determine if this explanation was plausible, the 13 kb insert was subcloned into pBluescript® SKII$^+$. Purified DNA was sequenced using primers corresponding to α_d I domain nucleic acid sequences 441-461, 591-612, 717-739, and reverse 898-918 in SEQ ID NO: 52. Sequence information was obtained using only the first 4441-4461 primer, and only the 5´-most exon of the I domain was efficiently amplified. The remainder of the I domain was not amplified. The resulting clone therefore comprised exon 6 of the mouse α_d gene, and intronic sequences to the 3´ and 5´ end of the exon. Exon 7 was not represented in the clone. After sequencing, a construct is generated containing neomycin resistance and thymidine kinase genes.

The neomycin resistance (neo^r) gene is inserted into the resulting plasmid in a manner that interrupts the protein coding sequence of the genomic mouse DNA. The resulting plasmid therefore contains a neo^r gene within the mouse genomic DNA sequences, all of which are positioned within a thymidine kinase encoding region. Plasmid construction in this manner is required to favor homologous recombination over random recombination [Chisaka, *et al.*, *Nature* *355*:516-520 (1992)].

An alternative strategy was used to generate constructs useful for production of α_d knock out mice. Two sets of oligonucleotide primers were submitted to Genome Systems, Inc. (St. Louis, MO) for high stringency PCR analysis of a large-insert library made from genomic DNA of embryonic stem cells. The primers corresponded to the first and last exons of the I domain in α_d. Three clones were identified, two of which, designated 1117 and 1118, were reactive with both primers and one, designated 1119, which only the primers from the last exon could amplify.

Identification of a Mouse Genomic α_d DNA

Plasmid DNA was prepared from bacterial lysates of clones 1117 and 1118 according to manufacturer's instructions (Genome Systems, Inc.) The α_d inserts were verified by PCR using the oligonucleotides madk.f1 (SEQ ID NO: 104) and madk.r1 (SEQ ID NO: 105) and madk.r2 (SEQ ID NO: 106).

madk.f1 SEQ ID NO: 104
TGT CCA GGA CAA GAG ATG GAC ATT GC

madk.r1 SEQ ID NO: 105
GAG CTA TTT CAT AGC AAG AAT GGG

madk.r2 SEQ ID NO: 106
TAT AGC ATA GCG AAT GAT CC

Aliquots of both plasmids were digested with restriction enzymes *Bam*HI, *Pst*I, *Sac*I, *Sal*I, *Sma*I, *Xba*I, and *Xho*I (Boehringer Mannheim). Each digest sample was resolved on 0.8% agarose gel and the polynucleotides transferred onto Hybond®-N^+ nucleic acid transfer membrane (Amersham) for analysis. The blot was probed with ^{32}P-random-primed DNA generated using a 1.6 kb template obtained by PCR using oligos madfor 1 (SEQ ID NO: 107) and madrev 1 (SEQ ID NO: 108).

madfor 1 SEQ ID NO: 107
ATG GTC CGT GGA GTT GTG ATC

madrev 1 SEQ ID NO: 108
TCG AGA TCC ACC AAA CTG CAC

Hybridization was carried out at 42°C overnight in SSPE buffer with 50% formamide. The labeled blot was washed five times in 2X SSPE at room temperature. Radiolabeled bands were visualized by exposure of the blot to Kodak X-Omat autoradiography film at -70°C for two hours.

Two fragments of interest were identified from clone 1118: a *Sac*I fragment of 4.1 kb and an *Xba*I fragment of 8.3 kb. The entire sample contents from the *Sac*I and *Xba*I digests of clone 1118 were ligated into the vector pBluescriptR KS^+

without further purification, and following ligation, the entire reaction contents were transformed into calcium-competent preparations of the *E. coli* strain TG1/lambda SmR. Resulting colonies were isolated and cultured overnight in 200 μl selective medium containing M13KO7 helper virus to replicate single stranded DNA. A 10 μl aliquot of supernatant from each well was then blotted onto Hybond®-N^+ transfer membrane and hybridized with the same probe and protocol as described above. Cultures were expanded from nine positive clones and plasmid DNA was isolated from each culture using a Wizard® Plus Miniprep DNA Purification System (Promega). Restriction digests and PCR were used to confirm presence and size of inserts in the isolated plasmids.

Three clones were subjected to sequence analysis using the vector primers T3 and T7 and oligonucleotide primers corresponding to murine α_d sequences. Sequence comparison of these three clones with the murine cDNA using Geneworks software indicated that all three contained both exons 1 and 2 of murine α_d. The longest clone, referred to as A, was an *Xba*I clone of 8280 kb length, and the two shorter clones, referred to as E and H, were identical *Sac*I clones of 4112 kb length. The 8280 kb *Xba*I clone was selected for further development.

Example 33
Cloning of Rabbit α_d

Construction and Screening of the Rabbit cDNA Library

Identification of human α_d homologs in rats and mice led to the investigation of the existence of a rabbit homolog which would be useful in rabbit models of human disease states described *infra*.

Poly A^+ RNA was prepared from a whole rabbit spleen using an Invitrogen FastTrack kit (San Diego, CA) according to manufacturer's suggested protocol and reagents supplied with the kit. From 1.65 g tissue, 73 μg poly A^+RNA were isolated. The rabbit spleen RNA was used to construct a ZAP® Express cDNA library using a kit from Stratagene (La Jolla, CA). Resulting cDNA was directionally cloned into *Eco*RI and *Xho*I sites in the lambda arms of a pBK-CMV phagemid vector. Gigapack® II Gold (Stratagene) was used to package the lambda arms into phage

particles. The resulting library titer was estimated to be approximately 8 x 10^5 particles, with an average insert size of 1.2 kb.

The library was amplified once by plating for confluent plaque growth and cell lysate was collected. The amplified library was plated at approximately 30,000 plaque forming units (pfu) per 150 mm plate with *E. coli* and the resulting mixture incubated for 12-16 hrs at 37°C to allow plaque formation. Phage DNA was transferred onto Hybond® N^+ nylon membranes (Amersham, Arlington Heights, Illinois). The membranes were hybridized with a mixture of two random primed radiolabeled mouse α_d PCR DNA probes. The first probe was generated from a PCR product spanning nucleotides 149-946 in SEQ ID NO: 52. The second probe was from a PCR product spanning nucleotides 2752-3651 in SEQ ID NO: 52. Probes were labeled by random priming (Boehringer Mannheim Random Primed DNA Labeling Kit) and the reaction mixture was passed over a Sephadex® G-50 column to remove unincorporated nucleotides. The hybridization solution was composed of 5X SSPE, 5X Denhardts, 1% SDS, 40% Formamide and the labeled probes at 1 x 10^6 dpm/ml. Hybridization was carried out at 42°C for 16-18 hours. Filters were washed extensively in 2X SSPE/0.1% SDS at room temperature and exposed to X-ray film to visualize any hybridizing plaques.

Two clones with significant sequence homology to human α_d were identified. Clone #2 was approximately 800 bp in length and mapped to the 5′ end of human α_d. Clone #2 includes an initiating methionine and complete leader sequence. Clone #7 was approximately 1.5 kb and includes an initiating methionine. The 5′ end of clone #7 overlapped that of clone #2, while the 3′ sequences terminated at a point beyond the I domain sequences. Clone #7 was completely sequenced by the primer walking method. The nucleotide and deduced amino acid sequences for clone #7 are set out in SEQ ID NOs: 100 and 101, respectively.

The predicted N terminal amino acid sequence for rabbit α_d as determined from clones #2 and #7 indicated a protein with 73% identity with human α_d, 65% identity with mouse α_d, and 58% identity with mouse CD11b, human CD11b, and human CD11c. The nucleic acid sequence for clone #2 is set out in SEQ ID NO: 92; the predicted amino acid sequence is set out in SEQ ID NO: 93

Isolation of a full length rabbit α_d cDNA was attempted using labeled rabbit clone # 7 and rescreening the cDNA library from which the fragment was derived. Twenty-five additional clones were identified with one, designated clone 49, determined to be the largest. Clone 49 was completely sequenced using the nested deletions technique. The nucleotide and amino acid sequences for clone 49 are set out in SEQ ID NOs: 102 and 103, respectively. Since clones #7 and #49 did not overlap, oligonucleotides were designed to be used as primers in a PCR with first strand rabbit spleen cDNA to isolate the missing sequence.

The relationship of the putative amino acid sequence of these two partial clones with that of other leukointegrins is described in Table 1.

Table 1.

Percent identity of β_2 integrin family members on the amino acid level.

	Human α_d	Rabbit #7	Rabbit #49
Human α_d	100	74	80
Mouse α_d	70	67	74
Rat α_d	70	66	73
Mouse CD11a	random*	28	28
Mouse CD11b	55	59	53
Human CD11a	36	28	28
Human CD11b	60	58	55
Human CD11c	66	59	62

* If <25% identity, it is just random alignment and not significant.

Isolation of a rabbit α_d clone allows expression of the protein, either on the surface of transfectants or as a soluble full length or truncated form. This protein is then used as an immunogen for the production of monoclonal antibodies for use in rabbit models of human disease states.

Example 34

Isolation of Monkey α_d

Preparation of affinity columns

In order to prepare an affinity column resin to isolate α_d from monkey spleen, 10 mg each of the anti-human α_d antibodies 212D and 217L were dialyzed overnight against coupling buffer containing 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3. Approximately 1.0 g of CNBr Sepharose® 4B (Pharmacia, Piscataway NJ) was prepared according to the manufacturer's recommended protocol and 1.0 ml of the resin combined with each of the dialyzed antibodies. The resulting slurry was mixed by rotation overnight at 4°C and coupled resin obtained by centrifugation for 5 minutes at 1000 rpm in a Beckman tabletop centrifuge. The nonabsorbed supernatant fraction was collected and assayed for the presence of uncoupled protein by spectrophotometer. Results indicated that all available antibody had bound to the gel matrix. Uncoupled active groups on the resins were blocked with 1 M ethanolamine for 2 hours at room temperature and the resins washed in a series of alternating high and low pH changes in Coupling buffer followed by a final wash using acetate buffer containing 0.1 M $NaC_2H_3O_2 \cdot 3H_2O$ and 0.5 M NaCl, pH 4.0. Both resins were stored at 4°C in Coupling buffer.

Preparation of Monkey Spleen

Female macaque spleens were obtained from the University of Washington's Regional Primate Center. Spleen tissue was injected with 100 U/ml collagenase D (Sigma) and minced into small pieces. Tissue pieces were then suspended in a small volume of Lysis Buffer containing 50 mM Tris, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 8.0, with 1.0% Triton-X100® detergent and stored at -70°C. Protease inhibitors PLA (a mixture of Pepstatin A, leupeptin, and aprotinin, each from Sigma) and 4-(2-aminoethyl) benzene sulfonyl fluoride-HCl (AEBSF) (Nova Biochem, La Jolla, CA) were added to prevent proteolysis of protein during storage. Tissue was stored until a total of six macaque spleens was obtained.

Spleen tissue from the six monkeys was pooled and homogenized in a Waring blender, three cycles of ten seconds each, in TSA Lysis Buffer containing 25 mM Tris, 0.15 M NaCl, 0.02% NaN_3, 1.0% Triton®, 1X PLA and 0.1 mM AEBSF. Lysate was collected and placed on a rocking platform for one hour at 4°C, and then

centrifuged for 15 minutes at 3000 rpm in a Beckman tabletop centrifuge. Supernatant was collected and the pelleted cellular debris discarded. A total volume of 550 ml of lysate was collected and precleared by incubating the lysate for 2 hours at 4°C with CNBr Sepharose® previously treated in 1 M ethanolamine in order to block reactive sites. Following incubation, the resin was removed by centrifugation and the supernatant collected.

Affinity Purification and Sequencing

The spleen lysate prepared as described above was divided in half and combined individually with the 212D- and 217L-prepared CNBr Sepharose® gels. The resulting slurries were mixed with rotation for three days at 4°C, after which the nonabsorbed fraction was collected by centrifugation at 10 minutes 1500 rpm in a Beckman tabletop centrifuge and saved. The gels were transferred to 15 ml centrifuge tubes and washed sequentially in several volumes of D-PBS. Aliquots of approximately 100 μl of each gel were boiled briefly in reducing sample buffer containing 0.1 M Tris-HCl, pH 6.8, 2.0% SDS, 20% glycerol, 0.0002% bromophenol blue, 10% β-mercaptoethanol (final concentration 5%) and loaded onto and proteins resolved using a 6.0% polyacrylamide SDS gel (SDS-PAGE). The gel was Coomasie stained and proteins having molecular weights consistent with α_d and CD18 were detected along with a number of background proteins.

In an attempt to improve purification of the protein having the molecular weight similar to α_d, two 100 μl aliquots of each gel with bound protein were washed by different means. In one method, gels were washed several times in buffer containing 150 mM NaCl, 10 mM Tris, 1.0% Triton-X100®, pH 8.0, and in a second method, gels were washed identically, but bound protein was eluted in a final wash with 0.05 M glycine, pH 2.4. As before, the eluted protein was boiled briefly in reducing sample buffer and resolved on a 6.0% SDS-PAGE gel. Coomasie staining detected only proteins consistent with α_d and CD18 from the resin washed in low pH glycine buffer, thus this isolation method was chosen. In order to isolate protein for sequencing, the remaining CNBr Sepharose® resin was washed four times as described above and approximately three quarters of the resin suspended in 2.0 ml 0.05 M

glycine, pH 2.4, and vortexed vigorously. The resin was pelleted by centrifugation for 3 minutes and the nonabsorbed fraction collected. The gel was then washed once more in glycine buffer and this wash pooled with the previous nonabsorbed fraction. The pooled fractions were dialyzed against D-PBS overnight at 4°C with two changes. After dialysis, the samples were dried down to reduce volumes to 1.0 ml.

For sequencing, the eluates were separated on 7.0% resolving gels and proteins transferred to Immobilon (PVDF) membranes (Millipore, Bedford MA) as described in Example 2. Briefly, the gels were washed once in deionized water and equilibrated for 15 to 45 minutes in 10 mM cyclohexylamino-propanesulfoic acid buffer (CAPS), pH 10.5, with 10% methanol. PVDF membranes were rinsed in both methanol and distilled water, then equilibrated in CAPS transfer buffer for 15 to 30 minutes. Proteins were transferred to PVDF membranes for 3 hours at 70 volts after which they were stained in filtered 0.1% R250 Coomasie stain for 10 minutes. Membranes were washed to destained in 50% methanol/10% acetic acid three times, 10 minutes each wash, washed once more in filtered water, and dried.

Two predominant protein bands of approximately 150 kD and 95 kD were detected from both the 212D- and 217L-coupled resins which were consistent with proteins detected on the previously run analytical scale gels. A less distinct band was observed on the membrane derived from 217L-coupled resin located directly beneath the protein at 150 kD, but the band was not detected after the membrane was dried. The 150 kD band from each membrane was excised from the membrane and directly sequenced with an Applied Biosystems (Foster City, CA) Model 473A protein sequencer according to the manufacturer's suggested method.

Results indicated that the amino terminal sequence of the monkey protein isolated using 212D-coupled resin had the amino acid sequence as set out in SEQ ID NO: 109, and the amino terminus of the protein isolated using 217L-coupled resin had the sequence shown in SEQ ID NO: 110. The "X" in SEQ ID NO: 110 indicates an indeterminable residue.

212D-Couple Protein SEQ ID NO: 109

NLDVEEPTIFQEDA

217L-Coupled Protein SEQ ID NO: 110

NLDVEEPTIFXEDA

A comparison of the monkey sequences with the amino terminal sequences of human α_d and other α chains in the β_2 integrin family is shown in Table 2.

Table 2

Comparison of Monkey α_d Amino Terminal Sequence With Human β_2 α Subunits

PROTEIN	SEQ ID NO:	AMINO TERMINAL SEQUENCE
Monkey α_d	111	F N L D V E E P T I F Q E D A
Human α_d	112	F N L D V E E P T I F Q E D A G G
Human CD11c	113	F N L D T E E L T A F V D S A G
Human CD11b	114	F N L D T E N A M T F Q E N A R G

Based on the sequence identify, it can be concluded that both 212D and 217L recognize α_d in both macaque and human.

Example 35

Characterization of 217L Antigen

Based on the N-terminal sequence of protein precipitated from monkey spleen in the previous example, it can be concluded that antibodies 217L and 212D recognize α_d protein in both monkey and human. Immunocytochemical (ICC) analysis and immunoprecipitation experiments, however, indicate that 217L has additional reactivity unshared by 212D. FACS and ICC experiments using antibodies to all α chains ruled out cross-reactivity of 217L with CD11c, the most closely related leukointegrin α chain, and CD11b. Therefore, it may be that the 217L antibody also recognizes either a conformational, glycosylation, or splice variant of α_d or a novel α chain which shares sequence homology with α_d.

The unique distribution of the antigen recognized by antibody 217L in sarcoid lung tissue (see Example 18), with a non-overlapping staining pattern in relationship to CD11c, suggests that the antigen may have biological significance. Therefore, in order to more fully understand the significance of the 217L antigen, analysis of the protein and underlying DNA encoding the protein are required and

various approaches are contemplated.

Immunoprecipitation of a protein complex from human dendritic cells or peripheral blood is carried out using the antibody 217L, followed by N-terminal sequence of the precipitated proteins. Sequence analysis will reveal whether the protein recognized on peripheral blood cells shares amino terminal identity with α_d. The protein precipitated from dendritic cells or peripheral blood cells is then treated with deglycosylating enzymes and compared to CD11c and α_d precipitated from other sources to provide a comparison of molecular weight of the primary amino acid sequence.

Additionally, a cDNA library generated from dendritic cell RNA is probed with the entire α_d cDNA under low stringency. Reactive clones are analyzed by nucleic acid sequencing over the entire length of the clone in order to determine if non-α_d sequences exist in the clones.

Example 36

Animal Models For Determining α_d Therapeutic Utility

Immunohistologic data in dog and *in situ* hybridization in rats and mice has determined that in spleen α_d is expressed primarily by macrophages present in red pulp and in lymph nodes, α_d is found in medullary cords and sinuses. The expression pattern is remarkably similar to what has been reported for two murine antigens defined by the monoclonal antibodies F4/80 and SK39. While biochemical characterization of these murine antigens has demonstrated that they are distinct from α_d, it is highly probably that α_d defines the same macrophage subset as the murine F4/80 and SK39 antigens.

In mouse, SK39-positive macrophages have been identified in splenic red pulp where they may participate in the clearance of foreign materials from circulation, and in medulla of lymph nodes [Jutila, *et al.*, *J.Leukocyte Biol.* *54*:30-39 (1993)]. SK39-positive macrophages have also been reported at sites of both acute and chronic inflammation. Furthermore, monocytes recruited to thioglycolate-inflamed peritoneal cavities also express the SK39 antigen. Collectively, these findings suggest that, if SK39$^+$ cells are also α_d^+, then these cells are responsible for the clearance of

foreign materials in the spleen and participate in inflammation where macrophages play a significant role.

While the function of α_d remains unclear, other more well characterized β_2 integrins have been shown to participate in a wide variety of adhesion events that facilitate cell migration, enhance phagocytosis, and promote cell-cell interactions, events which all lead to upregulation of inflammatory processes. Therefore, it is highly plausible that interfering with the normal α_d function may also interfere with inflammation where macrophages play a significant role. Such an anti-inflammatory effect could result from: i) blocking macrophage recruitment to sites of inflammation, ii) preventing macrophage activation at the site of inflammation or iii) interfering with macrophage effector functions which damage normal host tissue through either specific autoimmune responses or as a result of bystander cell damage.

Disease states in which there is evidence of macrophages playing a significant role in the disease process include multiple sclerosis, arthritis, graft atherosclerosis, some forms of diabetes and inflammatory bowel disease. Animal models, discussed below, have been shown to reproduce many of the aspects of these human disorders. Inhibitors of α_d function are tested in these model systems to determine if the potential exists for treating the corresponding human diseases.

Graft Arteriosclerosis

Cardiac transplantation is now the accepted form of therapeutic intervention for some types of end-state heart disease. As the use of cyclosporin A has increased one year survival rates to 80%, the development of progressive graft arteriosclerosis has emerged as the leading cause of death in cardiac transplants surviving beyond the first year. Recent studies have found that the incidence of significant graft arteriosclerosis 3 years following a cardiac transplant is in the range of 36-44% [Adams, *et al.*, *Transplantation 53*:1115-1119 (1992); Adams, *et al.*, *Transplantation 56*:794-799 (1993)].

Graft arteriosclerosis typically consists of diffuse, occlusive, intimal lesions which affect the entire coronary vessel wall, and are often accompanied by lipid deposition. While the pathogenesis of graft arteriosclerosis remains unknown, it is

presumably linked to histocompatibility differences between donor and recipient, and is immunologic in nature. Histologically, the areas of intimal thickening are composed primarily of macrophages, although T cells are occasionally seen. It is therefore possible that macrophages expressing α_d may play a significant role in the induction and/or development of graft arteriosclerosis. In such a case, monoclonal antibodies or small molecule inhibitors (for example, soluble ICAM-R) of α_d function could be given prophylactically to individuals who received heart transplants and are at risk of developing graft arteriosclerosis.

Although atherosclerosis in heart transplants presents the greatest threat to life, graft arteriosclerosis is also seen in other solid organ transplants, including kidneys and livers. Therapeutic use of α_d blocking agents could prevent graft arteriosclerosis in other organ transplants and reduce complications resulting from graft failure.

One model for graft arteriosclerosis in the rat involves heterotopic cardiac allografts transplanted across minor histocompatibility barriers. When Lewis cardiac allografts are transplanted into MHC class I and II compatible F-344 recipients, 80% of the allografts survive at least 3 weeks, while 25% of the grafts survive indefinitely. During this low-grade graft rejection, arteriosclerosis lesions form in the donor heart. Arterial lesions in 120 day old allografts typically have diffuse fibrotic intimal thickening indistinguishable in appearance from graft arteriosclerosis lesions found in rejecting human cardiac allografts.

Rats are transplanted with hearts mismatched at minor histocompatibility antigens, for example Lewis into F-344. Monoclonal antibodies specific for rat α_d or small molecule inhibitors of α_d are given periodically to transplant recipients. Treatment is expected to reduce the incidence of graft arteriosclerosis in non-rejecting donor hearts. Treatment of rats with α_d monoclonal antibodies or small molecule inhibitors may not be limited to prophylactic treatments. Blocking α_d function is also be expected to reduce macrophage mediated inflammation and allow reversal of arterial damage in the graft.

Atherosclerosis in Rabbits Fed Cholesterol

Rabbits fed an atherogenic diet containing a cholesterol supplement for approximately 12-16 weeks develop intimal lesions that cover most of the lumenal surface of the ascending aorta [Rosenfeld, *et al., Arteriosclerosis 7*:9-23 (1987); Rosenfeld, *et al., Arteriosclerosis 7*:24-34 (1987)]. The atherosclerotic lesions seen in these rabbits are simmer to those in humans. Lesions contain large numbers of T cells, most of which express CD45RO, a marker associated with memory T cells. Approximately half of the infiltrating T cells also express MHC class II antigen and some express the IL-2 receptor suggesting that many of the cells are in an activated state.

One feature of the atherosclerotic lesions found in cholesterol fed rabbits, but apparently absent in rodent models, is the accumulation of foam cell-rich lesions. Foam cell macrophages are believed to result from the uptake of oxidized low-density lipoprotein (LDL) by specific receptors. Oxidized LDL particles have been found to be toxic for some cell types including endothelial cells and smooth muscle cells. The uptake of potentially toxic, oxidized LDL particles by macrophages serves as an irritant and drives macrophage activation, contributing to the inflammation associated with atherosclerotic lesions.

Once monoclonal antibodies have been generated to rabbit α_d, cholesterol fed rabbits are treated. Treatments include prophylactic administration of α_d monoclonal antibodies or small molecule inhibitors, to demonstrate that α_d^+ macrophages are involved in the disease process. Additional studies would demonstrate that monoclonal antibodies to α_d or small molecule inhibitors are capable of reversing vessel damage detected in rabbits fed an atherogenic diet.

Insulin-dependent Diabetes

BB rats spontaneously develop insulin-dependent diabetes at 70-150 days of age. Using immunohistochemistry, MHC class II^+, $ED1^+$ macrophages can be detected infiltrating the islets early in the disease. Many of the macrophages appear to be engaged in phagocytosis of cell debris or normal cells. As the disease progresses,

larger numbers of macrophages are found infiltrating the islets, although significant numbers of T cells, and later B cells, also appear to be recruited to the site [Hanenberg, *et al., Diabetologia 32*:126-134 (1989)].

Development of diabetes in BB rats appears to depend on both early macrophage infiltration and subsequent T cells recruitment. Treatment of BB rats with silica particles, which are toxic to macrophages, has been effective in blocking the early macrophage infiltration of the islets. In the absence of early macrophage infiltration, subsequent tissue damage by an autoaggressive lymphocyte population fails to occur. Administration of monoclonal antibody OX-19 (specific for rat CD5) or monoclonal antibody OX-8 (specific for rat CD8), which block the T cell-associated phase of the disease, is also effective in suppressing the development of diabetes.

The central role of macrophages in the pathology of this model makes it attractive for testing inhibitors of α_d function. Rats genetically predisposed to the development of insulin-dependent diabetes are treated with monoclonal antibodies to α_d or small molecule inhibitors and evaluated for the development of the disease. Preventing or delaying clinical onset is evidence that α_d plays a pivotal role in macrophage damage to the islet cells.

Inflammatory Bowel Disease (Crohn's Disease, Ulcerative Colitis)

Animal models used in the study of inflammatory bowel disease (IBD) are generally elicited by intrarectal administration of noxious irritants (e.g. acetic acid or trinitrobenzene sulfonic acid/ethanol). Colonic inflammation induced by these agents is the result of chemical or metabolic injury and lacks the chronic and spontaneously relapsing inflammation associated with human IBD. However, a recently described model using subserosal injections of purified peptidoglycan-polysaccharide (PG-PS) polymers from either group A or group D streptococci appears to be a more physiologically relevant model for human IBD [Yamada, *et al., Gastroenterology 104*:759-771 (1993)].

In this model PG-PS is injected into the subserosal layer of the distal colon. The resulting inflammatory response is biphasic with an initial acute episode three days after injection, which is followed by a spontaneous chronic phase three to

four weeks later. The late phase response is granulomatous in nature, and results in colonic thickening, adhesions, colonic nodules and mucosal lesions. In addition to mucosal injury, PG-PS colitis frequently leads to arthritis anemia and granulomatous hepatitis. The extraintestinal manifestations of the disease make the model attractive for studying Crohn's colitis in that a significant number of patients with active Crohn's disease suffer from arthritic joint disease and hepatobillary inflammation.

Granulomatous lesions are the result of chronic inflammation which leads to the recruitment and subsequent activation of cells of the monocyte/macrophage lineage. Presence of granulomatous lesions in Crohn's disease and the above animal model make this an attractive clinical target for α_d monoclonal antibodies or other inhibitors of α_d function. Inhibitors of α_d function are expected to block the formation of lesions associated with IBD or even reverse tissue damage seen in the disease.

<u>Arthritis</u>

Arthritis appears to be a multi-factorial disease process involving a variety of inflammatory cell types including neutrophils, T lymphocytes and phagocytic macrophages. Although a variety of arthritis models exist, preparations of streptococcal cell wall proteoglycan produce a disorder most similar to the human disease.

In rats, streptococcal cell wall induces inflammation of peripheral joints characterized by repeated episodes of disease progression followed by remission and eventually resulting in joint destruction over a period of several months [Cromartie, *et al., J.Exp.Med. 146*:1585-1602 (1977); Schwab *et al., Infection and Immunity 59*:4436-4442 (1991)]. During the chronic phase of the disease, mononuclear phagocytes or macrophages are believed to play a major role in destruction of the synovium. Furthermore, agents which suppress the recruitment of macrophages into the synovium effectively reduce the inflammation and pathology characteristic of arthritis.

A central role for the macrophage in synovium destruction that leads to arthritis predicts that monoclonal antibodies to α_d or inhibitors of α_d function may have therapeutic potential in the treatment of this disease. As in other models previously

described, α_d monoclonal antibodies or small molecule inhibitors administered prophylactically are expected to block or moderate joint inflammation and prevent destruction of the synovium. Agents that interfere with α_d function may also moderate ongoing inflammation by preventing the recruitment of additional macrophages to the joint or blocking macrophage activation. The net result would be to reverse ongoing destruction of the joint and facilitate tissue repair.

Multiple Sclerosis

Although pathogenesis of multiple sclerosis (MS) remains unclear, it is generally accepted that the disease is mediated by $CD4^+$ T cells which recognize autoantigens in the central nervous system and initiate an inflammatory cascade. The resulting immune response results in the recruitment of additional inflammatory cells, including activated macrophages which contribute to the disease. Experimental autoimmune encephalomyelitis (EAE) is an animal model which reproduces some aspects of MS. Recently, monoclonal antibodies reactive with CD11b/CD18 [Huitinga, *et al., Eur.J.Immunol. 23*:709-715 (1993)] present on inflammatory macrophages have been shown to block both clinical and histologic disease. The results suggest that monoclonal antibodies or small molecule inhibitors to α_d are likely to be effective in blocking the inflammatory response in EAE. Such agents also have important therapeutic applications in the treatment of MS.

Immune Complex Alveolitis

Alveolar macrophages located in the alveolar ducts, airways, connective tissue, and pleural spaces of the lung represent the lung's first line of defense against inhaled environmental agents. In response to stimulation by agents, including bacterial-derived LPS, IFN-γ and immune complexes, alveolar macrophages release a variety of potent inflammatory mediators, including highly reactive oxygen radicals and nitrogen intermediates. While superoxide anions, hydrogen peroxide and nitric oxide (NO·) have important functions in eradicating pathogens and lysing tumor targets, these agents can have injurious effects on normal tissues.

In a rat model of immune complex alveolitis, NO· release from alveolar

macrophages has been shown to mediate much of the lung damage [Mulligan, *et al., Proc.Natl.Acad.Sci.(USA) 88*:638-6342 (1991)]. NO· has also been implicated as a mediator in other immune complex mediated injuries including dermal vasculitis [Mulligan, *et al., supra*] and could potentially play a role in diseases such as glomerulonephritis.

NO· mediated tissue damage is not limited to inflammation involving immune complexes. For example, microglial cell stimulated, by agents such as PMA, LPS or IFN-γ, produce NO· at levels capable of killing oligodendrocytes [Merrill, *et al., Immunol. 151*:2132 (1993)]. Pancreatic islet cells have also been found to be sensitive to NO·, and macrophage release of this mediator has been implicated in the tissue damage which leads to diabetes [Kroncke, *et al., BBRC 175*:752-758 (1991)]. More recently, it was conclusively demonstrated that NO· release plays a role in endotoxic shock [MacMicking, *et al., Cell 81*:641-650 (1995)]. When administered lipopolysaccharide (LPS), normal wild-type mice experience a severe, progressive decline in arterial pressure resulting in death. Mice deficient in inducible nitric oxide, however, experience a much less severe decline in arterial pressure in response to LPS, and all survive the treatment.

In vitro assays indicate that blockage of α_d is effective at blocking some aspects of macrophage (or leukocyte which express α_d, in general) activation, including NO· release. Alveolar macrophages stimulated with IFN-γ in the presence of anti-α_d polyclonal anti-serum (generated in rabbits against a rat α_d I domain polypeptide) were found to produce significantly less nitrite/nitrate - breakdown products of NO· than macrophages treated with control anti-serum. This finding indicates that monoclonal antibodies to α_d, particularly to the I-domain, may be potent anti-inflammatory agents with potential uses in MS, diabetes, lung inflammation and endotoxic shock. Furthermore, in contrast to CD18, which effects the function of a wide variety of leukocyte types, the limited distribution of α_d may make this a more attractive target than CD18 for preventing macrophage (or leukocyte which express α_d, in general) activation.

Rat IgG immune complex-induced alveolitis is a widely used experimental model important in understanding acute lung injury. The injury is elicited

by instilling anti-bovine serum albumin (BSA) antibodies into lungs via tracheal cannulation, followed by an intravenous injection of BSA. The formation of immune complexes in the microvasculature of the lung leads to complement activation and the recruitment of neutrophils into the lung. Presumably, formation of immune complexes in the lung following extravasation of leukocytes from the blood and subsequent leukocyte movement across lung epithelium. The subsequent release of mediators, including radicals, TNF-α and nitric oxide (NO·), from activated endothelial cells, neutrophils and macrophages which participate in progression of the disease. Pathologic features of the disease include increased vascular permeability leading to edema and the presence of large numbers of erythrocytes and PMNs present in the alveolar spaces.

Polyclonal anti-serum specific for the I domain of α_d was tested in a rat model of immune complex-induced alveolitis. The anti-α_d polyclonal serum was administered via tracheal cannulation at the same time anti-BSA was introduced into the lungs. Lung injury was subsequently elicited by intravenous administration of BSA along with a trace amount of ^{125}I-labeled BSA (approximately 800,000 cpm) to quantitate edema resulting from lung injury. Lung injury was allowed to proceed for four hours and damage was assessed using a lung permeability value, is defined as the ratio of ^{125}I-labeled BSA in the lung compared to the amount of label present in the 1.0 ml of blood. Typically lung permeability values for positive control rates range between 0.6 and 0.8, while negative controls (rats not receiving BSA) have permeability index values in the range of 0.1-0.2.

Initial studies indicated that treatment with anti-α_d polyclonal anti-serum reduced lung permeability values by greater that 50%, representing a dramatic moderation of lung injury. Historically, treatments with anti-CD18 have reduced permeability values by 60%. These findings indicate that α_d may be the most important β_2 integrin during acute lung injury, however it cannot be precisely determined if the effect of the anti-sera prohibits leukocyte extravasation from the blood, or movement across lung epithelia.

As additional proof that α_d moderates lung injury, TNF-alpha levels in the bronchoalveolar lavage fluid were evaluated. Treatment with the anti-α_d anti-serum

was found to reduce TNF-alpha levels approximately four-fold. TNF-alpha has long been viewed as an important mediator in acute lung inflammation, and responsible for the recruitment of inflammatory cells into sites of inflammation, cell activation and tissue damage. Presumably, anti-α_d anti-serum blocks activation of resident alveolar macrophages during the formation of immune complex alveolitis, and thereby moderates the release of TNF-α and NO·, and reduces subsequent tissue damage caused by these agents and the recruitment of neutrophils.

F344 Rat Model of LGL leukemia

LGL leukemia in the F344 rat was first described in the early to mid 1980's as a transplantable leukemia with stable NK cell activity. This leukemia has been suggested as a possible model for human T gamma lymphoma and T-cell Chronic Lymphocytic Leukemia [Ward and Reynolds, *Am. J. Pathol. 111*:1-10 (1982); Stromberg, *Am. J. Pathol. 119*:517-519 (1985); Reynolds, *et al. J.Immunol. 132*:534-540 (1984)]. This model provides abundant cells for studies of LGL and NK cell function. Of particular interest is the presence of α_d on the surface of these cells as detected using hamster anti-rat antibody 205C through FACS analysis described in Example 26. In view of this observation, the roles of α_d *in vitro* (for example, using the cytolytic assays previously described) and *in vivo* were examined.

The pathologic features of LGL leukemia include severe splenomegaly, a pale mottled liver, enlargement of peripheral lymph nodes and petechial hemorrhages in lung, brain, and lymph node. Because α_d is present in the red pulp of normal rat spleen (on splenic macrophages), and the hallmark of LGL leukemia is severe splenomegaly, it was hypothesized that the α_d positive NK tumor cells may also "home" or tether to a yet defined ligand and proliferate here. To test this hypothesis, tumor cells were radiolabeled and injected with and without α_d antibody treatment into recipient rats. Spleens from these animals were removed after three hours and the presence of NK tumor cells determined. A more complete description of the methods used and experimental results are as follows.

Tumor cells, obtained from the spleens of rats with LGL leukemia and prepared as described below, were adoptively transferred to recipient rats 2 to 4 weeks

prior to each experiment. From previous studies by histology and FACS analysis, it was known that a rapid proliferation of the tumor and resulting splenomegaly occurs about three to four weeks after adoptive transfer. In the first experiment, a spleen was removed from an animal that had been exposed to tumor cells for four weeks. A single-cell suspension was made by mincing up the spleen into smaller pieces with scissors and passing these pieces through a mesh screen in the presence of D-PBS. The cell suspension was collected in a 50 ml tube and centrifuged for 10 minutes at 1500 rpm in a Beckman tabletop centrifuge at room temperature. The supernatant was discarded, and the cells resuspended in 30 ml of D-PBS. Approximately 5.0 ml of this cell suspension was layered onto 5.0 ml of Histopaque, and the gradients centrifuged for 30 minutes at 1500 rpm. The cellular layer from these gradients was collected, pooled, and counted by hemacytometer. The cell number was adjusted so that each recipient rat received 1.0 x 10^7 cells, with a slight overestimation to account for cell loss during washes and preparing syringes. The cells were suspended in NK "test media" (RPMI-1640 plus antibiotics, plus 2% FBS) and labeled with 10 mCi of ^{51}chromium for one hour at 37°C. After incubation, the volume of the cell suspension was increased to 50 ml with test media and the cells pelleted by centrifugation for 10 minutes, 1200 rpm. The supernatant was discarded and the cells washed two more times as described. The labeled cells were suspended at a final concentration of 1 x 10^7 cells/ml and preincubated in the presence or absence of anti-rat α_d antibodies as well as a control IgG1 antibody prior to injection into recipient rats. The final concentration of antibody used per animal was adjusted to 5.5 mg/kg, or approximately 1 mg/animal. A minimum of four animals was used for each condition.

Recipient rats were weighed and injected subcutaneously with 150 to 200 μl ACE solution (containing 0.25 ml Ketamine, 0.2 ml Ace and 0.8 ml Rompin) to anesthetize. From each antibody treatment, 1.0 x 10^7 cells were injected intravenously into animals. Approximately 300 μl of each cell suspension was examined using a gamma counter to determine the total cpm injected/rat. The labeled NK cells were allowed to circulate in the rats for three hours after which the animals were sacrificed and 1.0 ml of peripheral blood drawn by aortic puncture. Spleens were removed from each animal, weighed, and counted using a gamma counter. To determine the

percentage of cells returning to the spleen, the counts per minute (cpm)/spleen were divided by the total known cpm injected into the rat. To determine the cpm in peripheral blood, an assumption was made that blood represents about 6.0% of the total rat's weight. The cpm in 1.0 ml blood was multiplied by 6.0% of the animal's total weight to determine total cpm in blood. This number was then divided by the total number of cpm's injected into each animal to obtain the percentage of cpm remaining in blood.

In the first experiment, antibodies 226B, 226G, 226H, 226I, 20C5B (a non-blocking CD18 antibody) and a control antibody were used. Antibodies 226B and 226G appeared to significantly reduce the number of cells returning to the spleen as compared to the control antibody and the other two 226 antibodies; approximately 7 to 8% of the labeled cells returned to the spleen after incubation with the control antibodies, while approximately 6% of the cells returned to the spleen after incubation with 226B and 226G antibodies. The percentage of total cpm in blood, between 0.9 and 1.4%, did not show a marked difference between treatment groups with the exception of 226B, which had lower values than all other groups.

In a second experiment, several adjustments were made to the protocol defined above. First, an increase to four animals per condition was made, and second, the spleen from a tumor-bearing rat was removed at 2.5 weeks post-adoptive transfer rather than four weeks as above. The NK cells were prepared in exactly the same manner and injected into recipient animals following incubation with either antibody 226B or 226G or a control antibody at the doses defined above. Again, the labeled cells were allowed to circulate for three hours after which the animals were sacrificed and blood and spleens collected as above. In addition, the tissues from two of the animals/condition were removed to determine other locations of the tumor cells. These tissues included liver, brain, thymus, lung, long bone (for bone marrow) and kidney.

The results indicated that, in the control IgG1, approximately 32% of tumor cells were in the spleen, whereas both 226B and 226G showed reduced numbers, 28% and 29%, respectively, of labeled cells in the spleen. The percentage of cells in the blood were similar for each antibody, approximately 3 to 4% of the total cpm's were found in blood, with 226G antibody treatment slightly less than the other two

groups.

The tissue distribution was similar between treatment groups with liver showing 27% of total cpm, brain 0.05%, thymus 0.10%, lung 15%, kidney 0.80%, and long bone 1.3%.

In a third experiment, an increase in the number of animal per condition (n=6 or 7) was made in an attempt to detect statistical differences between the three treatment groups above. Again, the spleen from an animal injected with tumor cells two weeks prior to the experiment was used and prepared by the same method described above. The cells were labeled in the same manner and injected into the animals and allowed to circulate for three hours. In this experiment, only blood samples and spleens were collected from animals due to the large number of animals used.

The results were consistent with the second experiment in that approximately 30% of labeled cells were observed to have returned to the spleen in the control group, while only 25% in 226B antibody-treated cells and 27% in 226G antibody-treated cells were found in the spleen. The blood values again did not show major differences between groups, with approximately 17% of the total cpm's found in blood in the control group and 15.8% and 14.75%, respectively. were found for 226B and 226G treated groups.

To determine if three hours was an optimum time point to examine differences between treatment groups, a small adjustment was made in a fourth experiment. Again, cells were isolated and prepared in the same way for injection into recipient animals, except that an additional anti-CD18 antibody 20C5B was added to the panel of test antibodies. In addition, only four animal were used for each condition. In this experiment, the cells were allowed to circulate for only 30 minutes after injection, at which point blood samples were drawn and spleen removed from the animals.

At the 30 minute time point, the total cpm in the spleen was reduced from values observed in the second and third experiments to 12 to 13%. There were no apparent differences between all treatment groups in the spleen samples, although two of the four animal in the group treated with antibody 226B did have slightly lower

values. The blood values were again similar between all groups, with approximately 6 to 7% of the total cpm found in blood. The only marked difference between blood groups was a larger spread in data points from 226B and 226G antibody-treated animals. These findings suggest that α_d plays a role in the homing of leukemia cells to the spleen. Experiments indicate that homing requires several hours and maximum inhibition with the α_d specific monoclonal antibodies occurs at 3 hours.

Macaque Models for Multiple Sclerosis and Atherosclerosis

Monoclonal antibodies 212D and 217L were shown by immunocytochemical staining and immunoprecipitation to cross-react with macaque splenocytes. The specificity of recognition was confirmed by immunoprecipitation and amino terminal sequencing of an α_d species homolog from macaque spleen (Example 34). In view of these previous observations, the two antibodies were used to stain tissues obtained from macaques in either experimental autoimmune encephalitis (EAE) or atherosclerosis studies. Both of these diseases are marked by infiltration into lesions of phagocytotic macrophages which take up myelin basic protein (MBP) in the EAE or low density lipoprotein in atherosclerosis. MBP or lipid-laden macrophages can be identified morphorologically or by staining with Oil Red O (ORO) or antibody Ham 56 (Dako, Carpinteria, CA). The protocol employed in these studies is as described in Example 18 to characterize α_d expression in human tissues.

Sections from macaque brains with EAE were marked by infiltration of lymphocytes and macrophages. Expression of α_d was localized to a subset of macrophages in lesions which stained with ORO indicating previous uptake of MBP. Lesions which were negative for ORO staining were also negative for α_d expression. This result suggested a direct correlation between ORO staining and α_d. Similar results were observed using antibodies 217K, 217I, and 217H.

Atherosclerosis lesions were obtained from either thoracic or abdominal arteries of macaques on high fat diets. Lesions occur in both locations in humans, but those which progress pathologically are more often located in the abdominal aorta. The lesions tested in this study were separated into five different stages (I through V) and normal. Stage (IV/V) lesions were derived from the abdominal aorta and the

remainder were derived from the thoracic aorta.

Early stage lesions (I/II) showed little macrophage infiltration and low or even absent levels of α_d expression. In later stage lesions, foam cell infiltration was greater and α_d expression was detectable.

Staining patterns for other leukointegrin α chain subunits were overlapping with, but not identical to, α_d expression in both tissues. Most notably, expression of α subunits of non-α_d leukointegrins was detected on lymphocytes that did not stain with anti-α_d antibodies.

These results suggest that α_d expression may be characteristic of phagocytotic macrophages in both animal models. It is unclear, however, whether α_d is directly involved in phagocytosis or some downstream process such as antigen presentation.

Example 37

Expression of α_d in Preclinical Models

In order to assess differential expression of α_d in various disease states, tissue sections from animal disease models were stained with anti-α_d polyclonal serum produced as described above (see Example 22). Tissue from normal and diseased rats was sectioned at 6 μm thickness and air dried on Superfrost Plus (VWR Scientific) slides at room temperature overnight. After drying, sections were stored at -70°C until use. Prior to use, slides were removed from -70°C and placed at 50°C for approximately 5 minutes. Sections were fixed in cold (4°C) acetone (Stephens Scientific) for 10 minutes at room temperature and allowed to dry at room temperature. Each section was blocked with 150 μl of a solution containing 30% normal rat serum (Harlan Bioproducts), 5% normal goat serum (Vector Laboratories) and 1% bovine serum (BSA) (Sigma Chemical Company) in 1X TBS for 30 minutes at room temperature, after which the solution was gently blotted from the sections. Rabbit polyclonal serum, at a protein concentration of 34 μg/ml, and preimmune serum from the same rabbit, at a protein concentration of 38.5 μg/ml, were diluted in the blocking solution and 100 μl separately applied to each tissue section for 30 minutes 37°C. The serum solution was blotted from the sections and unbound antibody removed by

washing three times in 1X TBS for 5 minutes. Excess TBS was removed by blotting following the final wash. Biotinylated goat anti-rabbit antibody from a Elite Rabbit lgG Vectastain® ABC kit (Vector) was prepared according to manufacturer's suggested protocol and 100 μl of the resulting solution was applied to each section for 15 minutes at 37°C. Slides were washed two times in 1X TBS for five minutes in each wash, after which 100 μl of streptavidin-gold conjugate (Goldmark Biologicals), diluted 1:100 in 5% normal rat serum and 1% BSA, was applied to each section for one hour at room temperature. Slides were washed three times with TBS for five minutes each wash, and 100 μl of 1% glutaraldehyde (Sigma) in TBS buffer was applied for five minutes at room temperature. Slides were again washed three times in TBS for five minutes each wash, and five times in sterile deionized water for three minutes each wash. Excess liquid was blotted from each slide and two drops each of silver enhancing and initiating solution (Goldmark Biologicals) were applied to each section. The reaction was allowed to proceed for 20-30 minutes at room temperature, after which the sections were rinsed thoroughly in sterile deionized water, air dried overnight at room temperature and mounted with Cytoseal 60 (VWR). As controls, tissue sections were labeled with monoclonal antibodies recognizing CD11a, CD11b, CD11c and CD18 in the same experiments by identical protocols.

Labeling with α_d polyclonal sera and monoclonal antibodies to CD11a, CD11b, CD11c, and CD18 revealed a staining pattern for α_d different from than observed for the other α subunits.

In normal lung tissue, α_d expression was detected on respiratory epithelium of the bronchi (but not the epithelium in the alveolar spaces) and on individual cells which appear to be alveolar macrophages within the airspaces. The signal observed with the polyclonal serum was significantly higher than the background signal level with the pre-immune serum control. In pulmonary granuloma tissue, 24 and 96 hours after administration of glycan, a different signal was detected with the α_d staining respiratory epithelium throughout the alveolar area and a stronger signal detected on what appear to be alveolar macrophages throughout the airways. In the lung tissue from animals which had presumably recovered from the disease (sacrificed 16 days after administration of glycan), no signal was observed with the α_d antibody.

Very little background was observed with the pre-immunization serum in each of these tissues.

Using rat lung tissue from an antigen-induced asthma model, a very strong signal was detected with α_d antibody in the respiratory epithelium of both the bronchi and the alveolar spaces. The signal was significantly higher than the background signal level in the pre-immunization serum control.

Preclinical Model - L. monocytogenes

Evidence suggests that α_d positive macrophages in the spleen red pulp are involved in the clearance of damaged rbcs and other particles from circulation. It is hypothesized that bacterial agents are also cleared from circulation by the α_d positive macrophages in the spleen red pulp. Non-infectious agents which would not require the induction of an antigen-specific T cell response would be eliminated directly by the red pulp macrophages. In contrast, opportunistic infectious agents cleared by the red pulp macrophage do require a product T cell immune response for the eradication of the bacteria. It was therefore proposed that α_d expression on red pulp macrophages may serve to regulate macrophage/T cell interactions either by regulating the movement of macrophages from the red pulp into the marginal zones or by acting as an accessory molecule involved in macrophage/T cell interactions leading T cell activation.

To investigate the role of α_d during immune responses to infectious agents, α_d expression is evaluated in the spleen using a murine model of *Listeria monocytogens*. Expression of α_d is examined on red pulp macrophages which have phagocytosed bacteria. Antibodies to α_d are also tested in the model to determine the role played by α_d in the induction of a protective T cell response to *L. monocytogenes*.

Example 38

The Role of α_d in Spinal Cord Injury

After central nervous system (CNS) trauma, the immune response involves a mixture of invading neutrophils, natural killer cells and phagocytic monocytes/macrophages [Means, *et al.*, *J. Neurophathol. & Exp. Neurol.*, *42*:707-719 (1983)]. This response includes the release of inflammatory mediators, induction of reactive microglia, infiltration of platelets, endothelial damage with enhanced vascular permeability and development of edema. Recent observations suggest that post-traumatic inflammation in the spinal cord contributes to chronic deficits, partly through demyelination or through more direct damage to neurons and axons [Blight, A.R., *Central Nervous System Trauma*, *2*:299-315 (1985)]. In addition, a recent study reported that the number of macrophages/microglia was significantly correlated with the amount of tissue damage at each level of the spinal cord following impact injury [Carlson, *et al.*, *Exp. Neurol.*, *151*:71-81 (1998)]. Both neutrophils and macrophages phagocytose debris which in turn induces an oxidative burst resulting in the production of reactive oxygen species. These antibacterial agents, although efficacious, can lead to damage in surrounding healthy tissue. Thus, it is possible that the infiltration of leukocytes and concomitant production of reactive oxygen species is involved with the spread of secondary injury beyond the initial impact site. This hypothesis is supported by studies in which blocking of neutrophil or macrophage infiltration led to decreases in the extent of injury following stroke or spinal cord injury [Blight, *Neurosci*, *60*:263-273 (1994)].

In order to assess the role of α_d in spinal cord injury, a rat model was utilized in combination with monoclonal antibodies to α_d, some of which block binding to its ligand VCAM-1. In this model, complete transection at the fourth thoracic (T) spinal cord segment is introduced, which consistently produces autonomic dysreflexia [Krassioukov, *et al.*, *Am. J. Physiol. 268*:H2077-H2083 (1995)]. This model is advantageous because the small surgical lesion produces a well-defined, narrow zone of primary tissue destruction that facilitates analysis of the effects of cord injury on the area.

All experiments were carried out in accordance with policies established in the "Guide to Care and Use of Experimental Animals" as prepared by the Canadian

Counsel on Animal Care. Forty-two male Wistar rats (Charles River) weighing 270-320 grams were initially administered atropine (0.5 mg/kg) and diazepam (2.5 mg/kg) by intraperitoneal injection. After ten minutes, the rats were anaesthetized by intraperitoneal injection with sodium pentobarbital (35 mg/kg). Supplemental injections of anaesthetic (2 mg/kg) were administered during surgery as necessary. The rats were placed on a heating pad during surgery and the body temperature was kept close to 37°C. The dorsal process of the third thoracic (T3) vertebra was removed and a laminectomy was performed to expose the spinal cord under microscopic guidance. The cord was completely transected at the T4 spinal segment with a scalpel blade. The muscles and skin above the laminectomy were closed and the animals recovered under a heat lamp. Postoperative care of the paraplegic rats was conducted as previously described [Krassioukov *et al., Neurosci. 70*:211-226 (1996)]. After recovery from surgery, food and water were provided *ad libitum*. Animals survived for two days following surgery.

The rats (four to five per group) were divided into the following treatment groups: (1) 1 mg/kg of either α_d monoclonal antibodies 226H, 236L, 226B, or an irrelevant isotype-matched IgG1 kappa antibody 1B7 and (2) 5 mg/kg of either α_d monoclonal antibodies 226H, 236L, or the control 1B7. Each mouse received only one antibody in the treatment regimen.

Antibodies were selected following *in vitro* binding assays using recombinant human VCAM-1 fused to an immunoglobulin region and a CHO cell line expressing rat α_d and human CD18. A panel of antibodies was examined for the ability of individual antibodies to block α_d binding to VCAM-1. Results from the binding assays indicated that some antibodies did not block binding ("non-blockers"), some block binding in the range of 50% ("medium blockers"), while others block binding in the range of 75% to 85% ("strong blockers). Representative antibodies from the non-blocking, medium blocking and strong blocking groups were chosen for use as described below.

All monoclonal antibodies were administered via tail vein injection on the day before surgery, immediately after surgery, and the following day. Antibodies were diluted in phosphate buffered saline, pH 7.2, without calcium chloride or magnesium chloride to ensure an adequate volume for ease of injection. In another control group, 15 mg/kg of methylprednisolone (MP) was injected via the trail vein at 30 minutes, 2

hours, and 24 hours following a complete transection of the spinal cord. An additional control group of rats had transected spinal cords as described above, but received no accompanying treatment.

Two days after surgery, the animals were deeply anaesthetized with an intraperitoneal injection of 3 g/kg urethane (Aldrich Chemical Company, Inc., Milwaukee, WI, USA) prior to transcardial perfusion. After opening of the thoracic cavity, heparin was injected into the left ventricle. The rats were perfused with 250 ml of oxygenated tissue culture medium, pH 7.4, (Dulbecco's modified Eagle medium; Gibco BRL) followed by 500 ml of 4% formaldehyde fixative in 0.1 M phosphate buffer (pH 7.4). The thoracic spinal cord caudal to the transection (T4-8) was removed for examination as described previously [Krenz and Weaver, *Neurosci 85*:443-458 (1998)]. Following overnight postfixation in the same fixative, the spinal cord portions were cryoprotected with 10%, 20%, and 30% sucrose solutions in PBS at 4°C. The spinal cord portions were then cut into horizontal sections (50 μm) on a cryostat. Sections were stained with 1% cresyl violet, pH 4, using standard procedures to visualize the polymorphonuclear leukocytes, cleared in xylene, and coverslipped with DPX mountant (BDH Laboratory Supplies, Poole, U.K.). The number of macrophages/microglia exhibiting a rounded, phagocytic morphology and neutrophils exhibiting the characteristic multilobed nuclei following cresyl violet staining were counted in the area of the cord at the lesion site. Quantitation of immune cells was performed using bright field microscopy and a 40X objective lens fitted with a grid (total area of 0.08 mm^2). Three sample areas, starting at the edge of the transected cord and moving caudally and from one lateral edge to the other were examined for immune cells. The procedure resulted in an average total quantified area of spinal cord of 2.72 mm^2. The process was then repeated in another spinal cord section. The sample areas were selected from horizontal sections of the cord with the largest grey matter (border between lamina V and VII) because the inflammatory response was most prominent in the grey matter. The total number of macrophages and neutrophils counted in each sample area was divided by the total sample area (mm^2) to acquire the mean number of macrophages and neutrophils per mm^2 in each treatment group. The antibody-treated groups (1 mg/kg and 5 mg/kg) were compared to the respective irrelevant IgG-matched control at the same dose and to a non-drug treated

transection control. In a further comparison, the groups most significantly lowering the mean number of immune cells were compared to MP treated animals and to surgery control animals. All cell counting was carried out blind with respect to the identification of the treatment group.

Results indicated that activated microglia and/or blood macrophages identified as mononuclear, round cells with large translucent cytoplasm were evident in the lesion site two days after transection . Control animals that received no accompanying α_d antibody or corticosteroid treatment were found to have an average of 396 ± 27 macrophage/microglia per mm^2 at the site of the cord injury. Treatment with the irrelevant IgG1 antibody 1B7 at the lower dose (1 mg/kg) increased the number of macrophage/microglia to 462 ± 46 per mm^2, but this value was not significantly different than control animals which received no antibody treatment. Of the α_d antibodies tested, 226H and 236L at 1 mg/kg each led to significant reductions in the average number of macrophages per mm^2 compared to the 1B7 antibody and to the transection control. Specifically, 226H administration reduced macrophage/microglia per mm^2 to 147 ± 17 and 236L administration reduced macrophage/microglia per mm^2 to 131 ± 8 compared to both 1B7 and animals receiving no treatment. In contrast, injection of α_d antibody 226B at 1 mg/kg reduced the number of macrophages/microglia per mm^2 to 327 ± 65, which was not significantly different from the control values. MP treatment led to an observation of 250 ± 24 macrophage/microglia per mm^2, which was significantly less than control animals but greater than that detected in animals treated with 226H and 236L α_d antibodies. This result was unexpected since methylprednisolone is the most widely used drug for clinical treatment of acute spinal cord injury.

When the dose of α_d antibodies was increased to 5 mg/kg, the average number of macrophage/microglia per mm^2 with 226H and 236L treatment was significantly lower than the control, but the number was not significantly different from the number in animals treated with the irrelevant IgG1 isotype matched control antibody.

The results with respect to neutrophilic leukocytes (NLs) indicated that the majority was detected either singly or in aggregates throughout the gray matter. Only a small portion of the visible NLs were detected in the white matter on each side of the grey matter. Moreover, some neutrophils were found adhering to the lumenal surface of

venules and arterioles in the spinal tissue sections. In animals that received no treatment, the average number of NLs per mm^2 was 295 ± 56 and in those treated with 1 mg/kg 1B7, the number of NLs increased significantly to 503 ± 93. No αd antibody treatment significantly reduced the number of NLs when compared to the control animals that received no treatment. Specifically, 226H and 226B at 1 mg/kg provided an increased number of NLs per mm^2 to 361 ± 80 and 332 ± 43, respectively. Compared to 1B7 treated animals, treatment with 236L at 1 mg/kg and MP both significantly decreased the number of NLs per mm^2 to 263 ± 47 and 193 ± 39, respectively. These observations, however, were not significantly different from the control group which received no treatment.

At 5 mg/kg, 1B7 treatment increased the average number of NLs to 343 ± 37, but the observed increase was not significant compared to the non-treated animal group. Compared to 1B7 treatment, antibody 226H decreased the average number of NL present to 236 ± 38, but the reduction was not significant compared to animals that received no treatment. In contrast, 236L reduced the number of NL to 190 ± 17 per mm^2 which was significant compared to animals receiving 1B7 treatment.

These results indicate that α_d monoclonal antibodies at low doses reduce the number of leukocytes in injured spinal cord, possibly through the disruption of an interaction with VCAM-1 which would be consistent with previously reported observations. Other reports using antibodies against the best known counterreceptor for VCAM-1, VLA-4, showed decreased infiltration of VLA-4 positive cells into the brain and prevention of clinical and pathological signs of experimental allergic encephalomyelitis (EAE). Similarly anti-TNFα treatment was shown to inhibit the incidence and severity of EAE and one mechanism of action was by inhibiting VCAM-1 expression on spinal cord vessels leading to significant reduction in leukocyte entry into the CNS. These previous observations suggest that blocking the interaction of α_d on the surface of leukocytes with VCAM-1 on the surface of endothelial cells or glial cells may be responsible for the observed attenuated inflammatory response.

Because one of the antibodies, 226H, that blocked macrophage infiltration did not block binding between α_d and VCAM-1, these results suggest that the mode of inhibition by the antibody can include blocking between α_d binding partners other than

VCAM-1. One possibility is the existence of a rat counterpart to ICAM-R. Previous observations have indicated that, in addition to VCAM-1, α_d also binds to ICAM-R, although with much less affinity that to VCAM-. Interestingly, ICAM-R appears to be absent on endothelial cells and is expressed primarily on resting monocytes, lymphocytes and neutrophils precluding its involvement in leukocyte-endothelial adhesion under normal circumstances. It has been suggested that ICAM-R has a role in the initial phases of leukocyte cell-cell contact and that ICAM-R is involved in the regulation of the LFA-1/ICAM-1 leukocyte intercellular interaction. The role of ICAM-R in the early leukocyte interaction has been shown to be induction of cell aggregation. Disruption of the initial contacts which give rise to aggregation reduces the effectiveness of the immune response. It has also been shown that the interaction of another ligand for ICAM-R, LFA-1 induces a switch of LFA-1 to its activated state at the intercellular contact site. It is possible that co-expression of α_d and ICAM-R on resting leukocytes could work in much the same way and that interaction between the two proteins may promote contact-dependent leukocyte activation events. Conversely, disruption of the interactions, for example, through use of an α_d antibody, could account for the reduced entry of leukocytes into the spinal cord.

Several explanations could account for the increase in macrophage infiltration. Most simply, the result may be a phenomenon of the system. Alternatively, the higher dose of antibody may have resulted in cross-linking of FcγR on the mature macrophages, resulting in an initial burst of chemokine production that attracted additional leukocytes to the injury site. In the same way, chemokine production could explain the greater numbers of both neutrophils and macrophages at the injury site after treatment with 1B7 that occurred in the majority of cases. Another possible explanation for the observed results is inter-animal variability since the α_d antibodies were tested in outbred strains of Wistar rats. For example, different groups of rats could have slightly different states of immunocompetence. In order to test this possibility, the experiments are replicated using both a low and high dose of the monoclonal antibodies at the same time in animals from the same or related litters.

Example 39
Expression of α_d in Crohn's Disease

Previous work (Example 18) indicated that leukointegrins are detected at higher levels in tissue sections from patients with Crohn's disease. In order to assess the degree to which α_d expression is modulated in Crohn's disease, expression was examined in tissue sections from diseased and normal colon as follows.

Colon tissue from five individuals with Crohn's disease and a normal colon were sectioned at 6 μm thickness and air dried on Superfrost Plus (VWR Scientific) slides for five minutes at room temperature. Slides were stored at -20°C until the assay was performed. Prior to use, slides were incubated at 50°C for approximately two minutes. Sections were fixed in cold (4°C) acetone (EM Science) for two minutes and allowed to air dry at room temperature. Sections were placed in buffer containing 100 ml 1X TBS, 1.1 ml 30% H_2O_2 (Sigma), and 1.0 ml 10% NaN_3 (Sigma) for 15 minutes at room temperature to remove endogenous peroxidase activity. Each section was incubated in 150 μl of a blocking solution containing 20% normal human serum (Boston Biomedica), 5% normal rat serum (Harlan), and 2% BSA (Sigma) in 1X TBS for 30 minutes at room temperature. After incubation, the solution was gently blotted from the sections. Primary monoclonal antibody was prepared at a protein concentration of 10 μg/ml in blocking solution and 75 μl was applied to each tissue section for one hour at room temperature. After incubation, sections were washed three times for five minutes each in 1X TBS to remove unbound antibody. Excess TBS was removed by aspirating around the tissue following the final wash. Biotinylated rat anti-mouse antibody (Jackson Laboratories) was diluted 1:400 in blocking solution and 75 μl was applied to each section for 30 minutes at room temperature. Slides were washed two times with 1X TBS for five minutes each wash. Peroxidase-conjugated avidin/biotin complex (Vector Laboratories) was prepared by adding 9 μl reagent A and 9 μl reagent B, both reagents supplied by the manufacturer, to 782 μl 1X TBS, and 75 μl of the resulting mixture was applied to each section for 30 minutes at room temperature. Slides were washed two times in 1X TBS for five minutes each wash. Substrate 3,3'-diaminobenzidine (DAB) (Vector Laboratories) was applied and color development was stopped by immersion in water. One drop of 1% osmic acid (VWR) was applied to each section for approximately 15 seconds to enhance the signal

intensity and the reaction was stopped by immersion in water. Sections were counterstained in Gill's hematoxylin #2 (Sigma) and rinsed in water before dehydrating and mounting with Cytoseal (VWR).

In the normal colon, no labeling was detected with antibodies 217L, 217K, or 212D. Antibody 240I labeled numerous cells in lymphoid aggregates as well as lymphocytes and eosinophils scattered in the lamina propria. Antibody 240I also labeled cell types that appeared to be either macrophages or activated lymphocytes. Staining with antibody 169A was similar to that of antibody 240I. Antibody 169B labeled lymphocytes and macrophages scattered in the lamina propria and submucosa, in addition to a subset of smooth muscle cells around arteries and in the muscularis externa.

With the Crohn's colon samples, the labeling patterns observed with the individual antibodies overlapped but the expression patterns were not identical. No labeling was detected with antibodies 212D or 217K. Antibody 240I labeled granulomas with differential expression on the multinucleated giant cells in the lymphoid aggregates and labeled lymphocytes in the lymphoid aggregates. Antibody 240I labeled eosinophils and lymphocytes scattered in the lamina propria. Antibody 217L also labeled granulomas with different expression on the multinucleated giant cells in the lymphoid aggregates. Antibody 217L labeled a small subset of lymphomas in the lamina propia and submucosa which were also labeled with 240I. The staining pattern of antibody 169A was very similar to that found for 240I except that 169A labeled fewer lymphocytes. Antibody 169B staining was similar to the 169A pattern except that 169B also labeled a subset of smooth muscle cells around the vessels and in the muscularis externa.

Example 40
TNFα Release from Rat Spleen α_d^+ Cells

In order to characterize a unique splenic subpopulation of α_d^+ cells with respect to the ability to produce cytokine upon stimulation, the following experiments were conducted.

Lewis rats were injected subcutaneously in the rear flank with 100 µl of a 1:1 emulsion of Complete Freund's Adjuvant (CFA) in PBS and the animals were sacrificed seven days later. The spleens were harvested and a single cell suspension was prepared by standard procedures. B cells, $CD4^+$ T helper cells, and macrophages were

selectively removed using monoclonal antibodies against CD4 (antibody W3/25, E.A.A.C.C. No: 84112002), CD11b (antibody OX42, E.A.A.C.C. N o: 87081803) and CD45Ra/b (antibody OX33, Pharmingen) pre-armed onto an anti-mouse IgG magnetic bead conjugate. The CD4 antibody identifies T cells, the CD11b antibody identifies macrophage, monocytes, granulocytes, and natural killer cells, and the CD45Ra/b antibody recognizes B cells, T cell subsets, monocytes, granulocytes, and macrophage. A magnet was then used to remove the antibody-coated cells. The non-adherent cells were collected and a positive selection was carried out using biotinylated rat anti-α_d monoclonal antibodies 205C and 226G, followed by incubation with Streptavidin magnetic beads. The antibody-coated cells were collected using a magnet and suspended at 5×10^5 cells/ml in growth media (RPMI 1640 including 2% normal Lewis rat serum, penicillin/streptomycin sodium pyruvate; L-glutamine).

Two ml of cell suspension was added to individual wells on a 24 well plate coated with 3 µg/ml anti-rat CD3 monoclonal antibody (G418, Pharmingen), irrelevant control antibody, or no antibody. The plate was incubated at 37°C in 7% CO_2 and supernatants from each well were collected after 20 hours, 48 hours, and 72 hours. Supernatants were aliquoted and stored at -70°C immediately upon collection. Prior to the assay, supernatants were diluted 1:2 and placed into an anti-rat TNFα detection assay (Biosource).

Results indicated that after stimulation with the anti-CD3 monoclonal antibody, the α_d^+ cells released approximately 280 pg/ml TNFα after 20 hours compared to approximately 40 pg/ml with the antibody control and media only treatment groups.

Example 41

Modulation of TNFα Release from Activated Splenocytes With α_d Antibodies

In order to assess the role of α_d^+ phagocytic splenocytes in an inflammatory response, the following experiments were performed.

Because it has previously been shown that α_d^+ splenic macrophages phagocytose magnetic particles injected into rats, cells of this type were collected in the following manner. Four mice were injected intravenously with 200 µl of a magnetic bead suspension (amine-conjugated, Perspective Biosystems). After 24 hours, spleens were removed and a single-cell suspension was prepared by passing the tissue through a wire-

mesh screen. The cells were isolated using a magnet and washed one time in PBS containing magnesium and calcium, placed into culture in RPMI/10% FBS medium, and grown under the following six conditions: (i) no treatment; (ii) with hamster anti-rat α_d monoclonal antibody 205C (10 μg/ml) that crossreacts with mouse α_d; (iii) with hamster anti-rat α_d monoclonal antibody 205E (10 μg/ml) which also crossreacts with mouse α_d; (iv) with lipopolysaccharide (LPS); (v) with LPS and monoclonal antibody 205C (10 μg/ml); and (vi) with LPS and monoclonal antibody 205E (10 μg/ml) that also crossreacts with mouse α_d.

Where indicated, the cells were first treated with the antibody for 30 minutes after which a 200 μl sample of conditioned medium was collected to represent the initial time point (t=0). LPS (10 ng/ml) was then added to wells as indicated above and aliquots of media were collected at 0.5 hour, 1 hour, 2 hours, and 4 hours and assayed for released TNFα by ELISA using a murine TNFα kit (ENDOGEN, #005452). Upon collection, samples were immediately frozen until assay. Just prior to assay, the conditioned media was diluted 1:1 and assayed according to the manufacturer's suggested protocol.

Results indicated that splenocytes which had not been activated with LPS showed no significant release of TNFα into the media regardless of prior antibody treatment. Splenocytes which had been treated with LPS released TNFα into the media at a detectable level, while the LPS-activated cells treated with either 205C or 205E antibody showed significantly lower levels of TNFα release. These results were consistent over all time points tested and confirmed in subsequently repeated assays. In addition, the same results were observed in later experiments with splenocytes that were not isolated using magnetic beads. Finally, preliminary results indicated that IL-1β release from splenocytes was similarly inhibited by the anti-α_d monoclonal antibodies.

Example 42
Characterization of α_d Expression on Eosinophils

Previous observations indicated that α_d is expressed on all peripheral blood eosinophils (Example 18). In order to further examine the expression and function of α_d on human eosinophils, the following analyses were carried out.

Expression of α_d integrin on human granulocytes

Expression of α_d on human granulocytes was examined on cells prepared as follows. Normodense eosinophils (*i.e.*, those with a normal specific gravity of greater than 1.09) were isolated from peripheral blood of allergic volunteers by density gradient centrifugation, hypotonic erythrocyte lysis, and immunomagnetic negative selection as previously described [Hansel, *et al., J. Immunol. Meth.145*:105 (1991)]. Neutrophils were purified from peripheral blood of normal volunteers by density gradient centrifugation and hypotonic erythrocyte lysis alone [Bochner, *et al., J. Immunol;. 145*:1832 (1990)]. Respective purities of the cell types always exceeded 95%. Enrichment of peripheral blood for basophils was performed using a double-percoll density gradient separation which increased the number of basophils to 3-10% of the total leukocyte count [Bochner, *et al, J. Immunol. Meth. 125*:265 (1989)]. Expression of integrins on the freshly isolated cells from blood following stimulation in culture was evaluated using single color indirect immunofluorescence and flow cytometry as previously described [Bochner, *et al., J. Immunol. Meth. 125*:265 (1989); Matsumoto, *et al., Blood 86*:1437 (1995)]. Dual color detection of basophils (using anti-IgE) was also performed. All samples were fixed in 0.1% paraformaldehyde (Sigma) and analyzed using an EPICS Profile II flow cytometer (Coulter). Approximately 10,000 events were collected and displayed on a four-log scale yielding values for means fluorescence intensity (MFI).

Results indicated that eosinophils express all four of the β_2 integrins. The level of surface expression of α_d integrin was greater than that of CD11c, but less than expression of α_4 integrin (CD49d), CD11a, or CD11b. Results also indicated that basophils have slightly higher levels of α_d integrin expression as compared to neutrophils.

Regulation of α_d integrin surface expression on human eosinophils

Initial studies were performed to determine whether eosinophils could rapidly mobilize intracellular stores of $\alpha_d\beta_2$ as has been reported for neutrophils [Van der Vieren, *et al., Immunity 3*:683 (1995)]. Purified peripheral blood eosinophils (prepared as described above) were incubated for 15 minutes with either PMA or the calcium ionophore A23187 and the surface expression of several α chains of the β_2 integrin family

was measured by indirect immunofluorescence (described above).

Results indicated that both PMA at 50 ng/ml and calcium ionophore at 1 μM significantly increased expression of α_d and CD11b. Within minutes of adding PMA, expression increased and reached significantly increased levels by ten minutes. This observation suggested that eosinophils have cytoplasmic stores of $\alpha_d\beta_2$ which, similar to CD11b stores, can be rapidly mobilized to the cell surface.

In view of these results, other eosinophil-active stimuli were tested for acute effects on $\alpha_d\beta_2$ expression. Incubation of eosinophils for fifteen minutes with MDC (100 nM), IL-5 (10 ng/ml), RANTES (100 ng/ml), and eotaxin (100 μM) failed to alter α_d integrin expression.

Previous observations have indicated that many eosinophil responses can be enhanced by prolonged exposure to certain cytokines, such as IL-5, in a phenomenon referred to as "priming" [Walsh, *et al., Immunol. 71*:258 (1990)]. Experiments were therefore designed to examine if priming eosinophil cultures with IL-5 would lead to changes in surface expression of α_d integrin. Purified eosinophils, prepared as described above, were incubated for four days with 10 ng/ml IL-5 and expression of various integrins assayed as described above.

Results indicated that while expression of α_d integrin on the cell surface increased four to five fold, the level of α_4 integrin expression remained unchanged. The kinetics of the increase in α_d integrin expression indicated a statistically significant increase in expression after four to seven days of culture. In contrast, levels of α_4 integrin did not change significantly. The kinetics of enhanced α_d expression with PMA exposure was similar to that of CD11b, suggesting that these two leukointegrins might exist in similar or identical intracellular compartments. The location of this compartment for either integrin in eosinophils is not known; however, in neutrophils, preformed stores of CD11b have been localized to specific granules [Todd, *et al., J. Clin. Invest. 74*:1280 (1984); Bainton, *et al., J. Exp. Med. 166*:1641 (1641)].

Because late phase bronchoalveolar lavage (BAL) eosinophils express many characteristics of cytokine-primed eosinophils [Kroegel, *et al.,Ann. Rev. Respir. Dis. 143*:A45 (1991); Sedgewick, *et al., J. Immunol. 149*:3710 (1992)], expression of α_d on this cell type was also examined. BAL cells were obtained from allergic patients who

had undergone an endobronchial segmental allergen challenge with either ragweed or *D. petrynissinus* extract 18 hours previously as described [Kroegel, *et al., J. Clin. Allergy Immunol. 93*725 (1994)]. Eosinophil purity in the late phase BAL fluid was 19 ± 4%.

Results indicated that late phase BAL eosinophils also showed a statistically significant increase in α_d integrin expression, with levels similar to those seen after three days of culture stimulated with IL-5. In examining levels of α_d integrin on late phase BAL eosinophils, *i.e.*, cells which have already undergone cell adhesion and migration to get to the airway lumen, levels of expression intermediate to those seen on freshly isolated and IL-5 cultured eosinophils were observed. These data suggest that at least a portion of the elevated levels of α_d found after IL-5 culture is likely due to increased transcription and translation of α_d integrin.

Eosinophils Expressing α_d Bind to VCAM-1

Although α_d integrin has been shown to bind ICAM-R and possibly mediate leukocyte-leukocyte adhesion [Van der Vieren, *et al., Immunity 3*:683 (1995)], experiments were designed to examine other possible ligands for α_d expressed on eosinophils. In part because of previous studies suggesting β_2 integrin-dependent, CD11b-independent eosinophil adhesion to VCAM-1 [Matsumoto, *et al., Blood 86*:1437 (1995)], initial studies were performed using immobilized recombinant VCAM-1.

For both freshly purified and cultured eosinophils, ^{51}Cr-labeled cell adhesion to VCAM-1 (250 ng/ml) or BSA (1%) coated wells was performed for 30 minutes at 37°C as previously described [Matsumoto, *et al., Blood 86*:1437 (1995)]. In some experiments, cells were preincubated for 30 minutes at 4°C with saturating concentrations of one or more of the following blocking monoclonal antibodies prior to examining adhesion: anti-CD18 (7E4), anti-CD11a (MHM24), anti-CD11b (clone 44), anti-CD11c (BU-15), anti-α_d (240I), and anti-α_4 (HP2/1).

For transfected and parental CHO cells, adhesion was performed using coated plates identical to those employed for eosinophil adhesion. Examination of the transformed CHO cells indicated that α_d expression was relatively low, and as a result, the interaction between CHO transfectants and VCAM-1 was not as strongly detected as that between eosinophils and VCAM-1. A modification of a previously described gentle

washing technique [Shanley, *et al., J. Immunol. 160*:1014 (1998)] was therefore employed. This technique allowed non-adherent cells to be dislodged from the inverted plate with centrifugation at 1 x *g* for 30 minutes at 20°C. Remaining adherent cells were then removed using 0.1 M EDTA (Sigma) and counted by flow cytometry. In addition to VCAM-1, E-selection (100 ng/ml) was also used to coat wells in some adhesion experiments. In addition to the blocking monoclonal antibodies used in the eosinophil studies, immobilized VCAM-1 was pretreated with an appropriate dilution of $F(ab')_2$ anti-VCAM-1 monoclonal antibody prior to the addition of CHO cells.

Results indicated that freshly isolated eosinophils adhered to VCAM-1 and monoclonal antibody blocking of α_4 integrin effectively inhibited adhesion. Blocking with the anti-CD11b antibody had no effect. Adhesion could also be significantly and consistently inhibited by anti-α_d monoclonal antibody 240I, albeit to a lesser degree (approximately 30% inhibition) than that observed using the anti-α_4 antibody .

Even more striking were results of VCAM-1 adhesion experiments in which IL-5 cultured eosinophils, expressing enhanced levels of α_d integrin, were employed. Under these conditions, monoclonal antibodies to CD18, α_d, or α_4 integrins were equally effective in reducing adhesion to background levels, while a combination of blocking antibodies to CD11a, CD11b, and CD11c had no effect. It was also observed that IL-5 cultured eosinophils displayed enhanced background adhesion and reduced VCAM-1 adhesion compared to that seen with freshly isolated eosinophils. Based on monoclonal antibody blocking studies with freshly isolated eosinophils, adhesion to VCAM-1 was mainly mediated through α_4 integrins. However, in IL-5-cultured eosinophils, adhesion to VCAM-1 was equally mediated by α_4 and α_d integrins. Together, these data are the first to demonstrate activation-dependent regulation of $\alpha_d\beta_2$ integrin expression and function on human eosinophils and document a novel function for $\alpha_d\beta_2$ as an alternative ligand for VCAM-1.

Based on the result that α_d integrins on eosinophils bind to VCAM-1 and can be upregulated with IL-5, this leuko-integrin may play a role in cytokine-primed eosinophil recruitment to inflammatory sites.

Example 43
CHO Cells Expressing α_d Bind VCAM-1

To further verify that $\alpha_d\beta_2$ functions as a ligand VCAM-1, CHO transfectants were generated expressing human α_d and β_2 integrin chains as follows.

Chinese hamster ovary cells were transfected as described in Example 11. The $\alpha_d\beta_2$-transfected CHO cells were cultured in DMEM/F12 media with 1 mM pyruvate and 2 mM L-glutamine (Biofluids) supplemented with 10% dialyzed FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 600 µg/ml G418 (all from Life Technologies). Media for culture of the parental CHO cell line was similar except that non-dialyzed FBS (Life Technologies) was used and 0.1 mM hypoxanthine and 16 nM thymidine (Sigma) were used in place of the G418. The transfected cells expressed α_d and β_2 integrin chains at modest levels and did not express CD11a, CD11b, CD11c, or α_4 integrins. The parental CHO cell line failed to express any of these integrins. Adhesion assays were performed as described above in Example 42.

Results indicated that the $\alpha_d\beta_2$-transfected CHO cells adhered to VCAM-1-coated wells. Adhesion was effectively blocked by an $F(ab')_2$ monoclonal antibody against the first domain of VCAM-1 as well as by monoclonal antibodies against either CD18 or α_d. In contrast, parental non-transfected CHO cells failed to adhere to VCAM-1 and neither cell type displayed significant adherence to wells coated with E-selectin.

The finding that the monoclonal antibody to the α_4 integrin binding site in the first domain of VCAM-1 completely blocked $\alpha_d\beta_2$ integrin dependent VCAM-1 adhesion strongly suggested that the $\alpha_d\beta_2$ binding site is near or identical to that for α_4 integrins. Since there is little amino acid homology between α_d and α_4 integrins, this result was unexpected. Whether $\alpha_d\beta_2$ integrins can bind to other α4 integrin ligands, such as fibronectin or mucosal addressin cell adhesion molecule-1, is unknown.

VCAM-1 Regions Required for αd Binding

The first two domains of VCAM-1 have been shown to support binding of α_4 integrins and relevant amino acids in those domains have been identified. In order to determine whether α_d shares similar sites of recognition in the VCAM-1 molecules, a plasmid was constructed containing sequences for domains 1 and 2 of VCAM-1 fused to

human immunoglobulin Fc. In addition, a modified version of the two domain VCAM-1 expression construct was generated by PCR to include a substitution mutation wherein alanine at residue 40 was replaced with an aspartate residue. The two expression constructs were transiently transfected into COS cells using the DEAE-Dextran protocol previously described. Protein was purified from the culture supernatant using Protein A Sepharose® as previously described.

The ability of CHO cells expressing either $\alpha_4\beta_1$ or $\alpha_d\beta_2$ to bind a five domain VCAM-1/Ig fusion protein or ICAM-1/Ig fusion protein was tested as follows. CAMs were immobilized on 96 well microtiter plates at 0.5 µg/well in bicarbonate buffer (pH 9.5). Plates were blocked with 1% fish gelatin and treated with either buffer, irrelevant antibody or a blocking VCAM-1 antibody. The α_d or α_4 transformed CHO cells were treated with either buffer only, irrelevant antibody of a monoclonal antibody specific for the alpha chain (130K or 217I for α_d or α4.1 for α_4) or a blocking alpha chain antibody. Cells were washed before addition to the CAM-coated wells at a density of 100,000 cells per well. Cells were incubated in the presence of the immobilized antibody for twenty minutes before addition of 5% glutaraldehyde. Following fixation, plates were washed with distilled water and cells were stained with 1% crystal violet, After destaining with 66% ethanol for several hours, absorbance at 570 nm was measured on a Dynatech plate reader.

Results indicated that both $\alpha_d\beta_2$ and $\alpha_4\beta_1$ recognized the five domain and two domain forms of VCAM-1 to equivalent degrees indicating that additional domains might not be required for binding. While differences were detected between the α_d/VCAM-1 binding and α_4/VCAM-12 binding, it is likely that the differences resulted from differential expression in the transformed CHO cells. Binding of both cell lines was blocked by the VCAM-1 antibody (50 to 100%), α_4 antibody (100%) and α_d antibody (50%). The α_4 transfected line did not recognize the mutant VCAM-1 and binding of the α_d cell line to the mutant was 50% of that detected with wild-type VCAM-1. Neither CHO cell line exhibited binding of ICAM-1/Ig. Combined, these results suggest that both α_d and α_4 recognize domains 1 and 2 of VCAM-1 and recognize overlapping, but not identical, epitopes.

Example 44
Targeting α_d as a Tumor Antigen

Spatially and temporally restricted expression of α_d suggests that the molecule may serve as a target for removal of pathogenic cell populations that express α_d on the surface. Several previous observations lead to this possibility.

For example, compared to other leukointegrins, α_d expression is less widespread. Expression of α_d appears to be limited to a specialized and/or highly differentiated subset of leukocytes. Unlike other leukointegrins, α_d expression appears to be subject to regulation as evidenced by rapid down regulation on primary or transformed cells in culture. Monoclonal antibodies to α_d show variable reactivity even though the antibodies are specific for α_d. For example, the anti α_d antibody 212D shows limited reactivity with normal tissue and highly differentiated myeloid cells as compared to reactivity observed with another anti-α_d monoclonal antibody 217L. Interestingly, knockout α_d mice survive until birth and beyond, but this observation provides little information relating to the biological function of α_d. Finally, α_d expression has been detected on an estimated 70% of canine leukemias, and high levels of α_d expression have been detected on freshly isolated NK leukemia cells from F344 rats (Example 26). While tumor cells would be a preferred population for clearance using α_d as the target, any undesirable cell type that expresses α_d might also be cleared in the manner described below.

One or more antibodies are selected with preference for those exhibiting low level reactivity with normal tissue. One possibility would be antibody 212D for reasons discussed above. Appropriate control antibodies are also selected, including an irrelevant antibody as negative control. Blood and tumor samples are obtained from leukemia and lymphoma patients and screened by immunocytochemistry and for cell surface expression with analysis by FAScan, immunoprecipitation and Western blot analysis using the selected antibodies. Detection of positive staining would then lead to alternative procedures in developing the clearance method.

In one approach, the hypervariable region of the positive staining antibody selected above is cloned and expressed in the context of a complement-fixing human isotype. After subcloning and isotype switching, specification and reactivity is again assessed as described above including, for example, FACS analysis, histology on normal

tissue, and immunoprecipitation. A cassette vector was developed for the purpose of expressing a chosen hypervariable region in a human IgG1 context. In addition, a series of primers were designed and synthesized to facilitate amplification of a hypervariable region of an antibody of interest from a hybridoma cell line [Gavilondo-Cowley, *et al.*, in HYBRIDOMA, Vol 9 No; 5, 1990, Mary Anne Leibert Inc., Publishers, Media, PA, pp.407-417]. The resulting antibody is then tested *in vitro* to determine if its binding in the presence of complement results in cell death. Preferably, the *in vitro* assay is carried out using tumor cells. Control assays will determine if the monoclonal antibody exhibits the same activity in cell cultures that do not express α_d.

The monoclonal antibody is also tested to determine if binding results in internalization which indicates that the antibody can be conjugated with a cytotoxic drug.

A preclinical model is developed wherein *in vivo* cytolytic activity is examined. In one example, equivalency is examined in the F344 rat model (Example 26). Another model is the SCID/Hu system wherein human cells have been transplanted. For example, myeloid U937 cells, Jurkat T cells, or human colon carcinoma HTC166 cells are transplanted into mice, tumors are harvested, and the tumors are stained for surface antigens using anti-α_d antibodies (*e.g.*, 212D and 217L). Detection of α_d expression leads to use of the antibodies *in vivo* to remove tumors.

Example 44
Human Anti-α_d Monoclonal Antibodies

Human monoclonal antibodies are identified by screening antibody repertoires displayed on filamentous phage as previously described [Waterhouse, *et al.*, *Nucl. Acids Res. 21*:2265-2266 (1993); Parsons, *et al.*, *Protein Engineering 9*:1043-1049 (1996)]. Briefly, functional V-gene segments from non-immunized human donors are used to construct a repertoire of single-chain Fv (scFv) fragments displayed on the surface of phage. Fragments are cloned in a phagemid vector which permits both phage displayed and soluble scFv to be produced without subcloning. A histidine tag has been incorporated to allow rapid purification of scFv by nickel chelate chromatography. Use of this library format generally permits isolation of human monoclonal antibody fragments in less than two weeks. Isolation is carried out as previously described [Marks, *et al.*, *J. Mol. Biol. 222*:581-597 (1991), Vaughan, *et al.*, *Nature Biotechnol. 14*:309-314 (1996)].

Preferably antibodies are identified that specifically recognize the I domain

Example 45
Anti-α_d Antibody Treatment in Motheaten Mice

In motheaten mutant mice [Koo, *et al., J. Immunol. 147*:1194-1200 (1992); Koo. *et al., J. Immunol. 151*:6733-6741 (1993)], the autosomal recessive me^v gene occurs spontaneously as a point mutation of the hematopoietic cell protein tyrosine phosphatase in C57BL/6 mice. Homozygotes develop a chronic myelomonocytic inflammation involving accumulation of myelomonocytic cells in the lung and skin which results in interstitial pneumonitis, thymic atrophy, and T cell and NK cell dysfunction. The inflammatory condition is transferable by bone marrow cell of these mice indicating that the me^v mutation is due to a stem cell defect in the myelomonocytic pathway. Since α_d is present in myeloid cells, a procedure was undertaken to assess any inhibitory effects on the immunopathologic changes with anti-α_d monoclonal antibody treatment in normal mice following bone marrow transplant from motheaten mutant mice.

C57BL/6J (B6)-me^v/me^v and their normal +/-siblings (B6)-+/- mice were obtained from the Jackson Laboratory, Bar Harbor, ME. Mice were maintained in a pathogen free environment with food and water provided *ad libitum*. All mice were six to ten weeks old.

At Day -1, all mice received 2 µg/ml α-NK1.1 antibody, PK136, Pharmingen) in 0.5 ml PBS via intraperitoneal injection. At Day 0, all B6 +/- mice were irradiated with 750 Rad. Bone marrow was harvested from (B6) me^v/me^v as previously described. Cells removed from tibia and femur were transferred to supplemented RPMI culture media and incubated for two hours prior to intravenous injection into the irradiated mice. Mice were immediately treated with either anti-α_d antibody 205C or irrelevant antibody at 5 mg/kg in 200 µl PBS via intraperitoneal injection. Purified 205C hamster anti-rat cross-reactive to mouse α_d monoclonal antibody has been described above. As a negative control, one group of mice received an equal volume saline injection. On Days 4 through 25, mice were treated with either antibody or saline injection every other day for a total of ten treatments and animals were monitored for changes in weight and signs of disease. In general, observations for each group were continued for a total of two months.

A moribund state is the endpoint in the assay. Animals that become very sick, however, are sacrificed. Survival rate among the groups are assessed and histological analyses of tissue are used as additional indicators of the efficacy of α_d antibody treatment. A similar study looking at the therapeutic properties of α_d antibodies is conducted to complement the prophylactic study described above.

As of day 35, none of the mice treated with the α_d antibody had dies, while two of the nine mice in the saline treated group had died and three of the remaining seven were developing conditions typical of the syndrome. In the group treated with irrelevant antibody, three of the eight had died.

Example 46
Expression of α_d on Human Leukemias

Leukemias can be divided into two classes, myeloid and lymphoid, according to cell lineage and both of these classes can be further distinguished as acute or chronic. Because of the apparent restriction of α_d expression to myeloid lineage cells, it was hypothesized that myeloid, but not lymphocytic, leukemias express α_d. A second line of inquiry, if the first hypothesis was correct, was to determine if α_d expression varied according to pathogenicity, thereby implying a disease-related function for α_d on these cells.

Expression of α_d on peripheral blood cells has been detected using antibodies 212D and 217L as described in Example 18. In the examination of leukemia cells, normal bone marrow cells were first analyzed by flow cytometric methods for α_d expression to establish a baseline for this cell type. Antibodies 212D and 217L were used to stain patient samples according to standard protocols and both antibodies exhibited weak reactivity with monocytes in the marrow. Antibody 212D was only marginally positive.

Flow cytometric analysis of leukemic cells from either peripheral blood or bone marrow of patients indicated the presence of 212D and 217L epitopes on myeloid blasts and monoblasts in three actue myelogenous leukemia (AML) patients. Expression was also observed on cells from a patient with chronic lymphocytic leukemia (CLL). Cells from another AML patient were evaluated at a later date and found to be α_d positive. Expression of α_d on the cells was 50 to 100% higher than control monoclonal antibodies,

but significantly lower than CD11a, CD11b, and CD11c expression levels.

In light of these results, the U937 cell line, a myeloid lineage leukemia equivalent to stage M-4 (on a differentiation scale of M1-M5) AML cells, was also evaluated. The expression patterns of CD11a, CD11b, CD11c, and α_d were similar to those of AML patient cells. Interestingly, the presence of cell-surface α_d protein was dependent on culture conditions. Rich medium with high serum levels (Iscove's Modified Dulbecco's Medium, 20% FBS) supported α_d expression, while basic culture medium (RPMI, 10% FBS) did not.

The finding that α_d expression was detected on lymphoblasts from a CLL patient indicates that α_d can be expressed in lymphocyte-lineage cells and is consistent with other data (*i.e.*, α_d expression on rat $CD5^+$ cells and canine $CD8^+$ cells). The relatively high level expression of other leukointegrins on these cells would preclude use of these cells to examine α_d function in a reproducible fashion, and suggests that the functional redundancy of this family would compensate for inhibition of one member in these cell types. In fact, α_d expression by these cells may be coincidental to aberrant transcription.

While these experiments did not fully support the initial hypotheses, the presence of α_d protein on both myeloid and lymphoid lineage leukemias suggests that a broad patient population may benefit from use of anti-α_d therapies aimed at tumor removal rather than functional inhibition.

Numerous modifications and variations in the invention as set forth in the above illustrative examples are expected to occur to those skilled in the art. Consequently only such limitations as appear in the appended claims should be placed on the invention.